SAFETY OF ISOPROPYL CITRATE

AND
ISOPROPYL CITRATE MIXTURE

FOR INCLUSION IN THE SCIENTIFIC LITERATURE

REVIEW ON CITRATES PB 223 850

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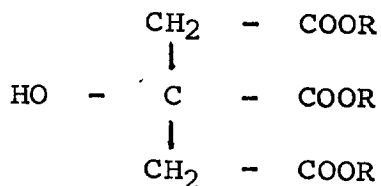SUBMITTED BY

CPC INTERNATIONAL INC.

JUNE 11, 1974

PROPOSED SPECIFICATION FOR

ISOPROPYL CITRATE (2).

Chemical Name Mixed citric acid esters of 2-propanol

Structural Formula

$$
\begin{array}{ccc}
 & CH_2 & - \quad COOR \\
 & | & \\
HO - & C & - \quad COOR \\
 & | & \\
 & CH_2 & - \quad COOR \\
\end{array}
$$

Where one R in the major component (mono-isopropyl citrate) is isopropyl and the remainder is hydrogen.

DESCRIPTION

Chemical Description Approximate composition of isopropyl citrate.

Monoisopropyl citrate (major component) –
  25 parts by weight.
Diisopropyl citrate – 9 parts by weight.
Triisopropyl citrate – 4 parts by weight.

The article of commerce conforms to the following specifications: Isopropyl citrate mixture is composed of approximately 38 parts by weight of isopropyl citrate (distribution of esters indicated above) in 62 parts by weight of mono- and di-glycerides and is an oil miscible semi-solid material. The commercial product may be further specified as to saponification value, acid value, citric acid and isopropyl content.

Appearance Isopropyl citrate is a viscous, colorless syrup exhibiting some crystallization upon standing.

FUNCTIONAL USES Antioxidant, sequestrant.

CHARACTERISTICS

Identification Tests

A. <u>Solubility</u>

<u>Water</u>:   soluble.
Ethanol: soluble.

B. Reflux 2 g of material with 50 ml of sodium hydroxide TS for one hour. Distill off 20 ml. Place 8 g chromic oxide in a flask, add 15 ml water and 2 ml concentrated sulfuric acid. Provide the flask with a reflux condenser and add 5 ml distillate slowly through the condenser. Reflux for 30 minutes, then cool and distill off 2 ml. Add 3 ml water and 10 ml mercuric sulfate TS to the distillate. Heat in boiling water bath for three minutes. A white or yellow precipitate within three minutes indicates the presence of isopropanol.

C. Reflux 3 g of material with 50 ml of sodium hydroxide TS for one hour and stand to cool. This solution is used for the following tests.

(i) Neutralize the solution with diluted sulfuric acid (1 in 20), add an excess of mercuric sulfate TS, heat to boil, and add potassium permanganate TS. The colour of the solution disappears and a white precipitate is produced.

(ii) Neutralize the solution with hydrochloric acid, add an excess of calcium chloride TS and boil. White crystalline precipitate, which is insoluble in sodium hydroxide TS but soluble in dilute hydrochloric acid TS, is produced.

Purity Tests   Acids, other than citric acid, and
                                alcohols other than 2-propanol, should
                                be absent.

                                * Sulfated ash: Not more than 0.3%.

                                * Arsenic: Not more than 1 mg/kg.

                                * Lead: Not more than 10 mg/kg.

                                * Heavy metals: Not more than 30 mg/kg.

                        * See General Methods.

ISOPROPYL CITRATE MIXTURE

DEFINITION

Isopropyl citrate mixture, used as a sequestering agent in anti-
oxidant mixtures and in fatty foods, is defined by the Joint
FAO/WHO Expert Committee on Food Additives in its Sixth Report
(1) as "A mixture consisting of approximately 27% monoisopropyl
citrate, 9% diisopropyl citrate, 2% triisopropyl citrate, and
62% mono- and diglycerides.

Subsequently, the modified specification given in this report,
reflecting and composition of isopropyl citrate mixture as actually
used, was proposed to the Joint Expert Committee (2).

DIGESTIBILITY

The digestibility of isopropyl citrates was studied in the early
fifties by Deuel and his co-workers at the University of Southern
California at Los Angeles. Incorporation of isopropyl citrate
esters in amounts as high as 10% of the diet of rats or dogs re-
sulted in almost complete absorption of the esters without lowering
the digestibility of the dietary fat (in this case, margarine) (3).

TOXICITY

Deuel and his co-workers determined acute toxicity of isopropyl
citrates in rats and dogs, conducted short-term feeding studies
in rats, rabbits and dogs, and long-term feeding studies in rats.
No deleterious effects were observed in any of the studies (4).

The biological data supplied in the Sixth Report of the Joint
FAO/WHO Expert Committee on Food Additives (1) were as follows:

BIOLOGICAL DATA

Acute Toxicity

Animal	Route	LD50 (mg/kg body weight)	Reference
Rat	oral	2800-3700	1
Dog	oral	2250	1

Short-term studies

Rat

A group of rats were fed isopropyl mixture in the diet at the
rate of 1500-2000 mg/rat per day for 6 weeks. There was no
demonstrable effect on growth or mortality and there were no
pathological findings.[1]

Rabbit

A group of rabbits were fed isopropyl mixture at an average level
of about 3600 mg/rabbit per day for 6 weeks. There was no demon-
strable effect on growth or mortality and there were no abnormal
findings post mortem.[1]

Dog

A group of dogs were fed isopropyl mixture in the diet at the level
of 0.06% for 6 weeks with no demonstrable effect on growth or
mortality and no pathological findings.[1]

Long-term studies

Rat

Groups of rats were fed isopropyl mixture in the diet at the levels
of 0%, 0.28%, 0.56% and 2.8% for a period of two years. No dele-
terious effects were noted in any of the treated groups with respect
to growth rate, mortality or histopathology of the tissues.[1]

1. Deuel, H.J., Greenberg, S.M., Calbert, C.E., Baker, R. &
    Fisher, H.R. (1951) Food Res., 16, 258.

Multigeneration studies likewise indicated that isopropyl mixture at a level of 2.8% in the diet was innocuous.[1]

Biochemical aspects

Studies in the rat showed that isopropyl mixture was readily absorbed when it was incorporated in the diet up to the 10% level.[2]

Comments on the experimental studies reported

No deleterious effects were observed in the short-term studies in rats, rabbits and dogs, or in the long-term studies in rats.

EVALUATION

Level causing no significant toxicological effect in the rat

2.8% (=28,000 ppm) in the diet, equivalent to 1400 mg/kg body weight per day.

Estimate of acceptable daily intakes for man

	mg/kg body weight
Unconditional acceptance	0-7
Conditional acceptance	7-20

1. Deuel, H.J., Greenberg, S.M., Calbert, C.E., Baker, R. & Fisher, H.R. (1951) Food Res., 16, 258.

2. Calbert, C.E., Greenberg, S M., Kryder, G. & Deuel, H.J. (1951) Food Res. 16, 294.

Isopropyl citrate was one of a number of substances reevaluated
by the Joint Expert Committee in 1966. In its Tenth Report (5),
it gave the same values as in its Sixth Report for unconditional
and conditional intake levels.

In 1973, the Joint Expert Committee revised its Acceptable Daily
Intake statement contained in the Seventeenth Report (6) to read:

> "Isopropyl citrate mixture and monoisopropyl citrate.
> On the basis of the no-effect level in the rat, the ADI
> for man was estimated at 0-14 mg/kg body weight."

U.S. FOOD AND DRUG ADMINISTRATION STATUS

Isopropyl citrate is listed as generally recognized as safe as a
sequestrant with a tolerance of 0.02% (7).

USES

Reports in the technical and patent literature document the use
of isopropyl citrate in the stabilization of oils (8,9,12-24);
for flavor retention of cheese (26); in the stabilization of de-
hydrated food products (27), of egg products (27-29), of margarine
(10,25), of milk fats (11), of nut meats (30), and of potato chips
(31); and to improve the whipping properties of gelatin and gelatin-
containing products (32).

LIST OF REFERENCES - MATERIALS ATTACHED

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        228 (1962).

                as modified by proposed changes submitted in

(2)   E. L. Sexton (Best Foods - CPC International Inc.) to
        R. K. Malik (Joint FAO/WHO Expert Committee on Food
        Additives), March 11, 1974.

(3)   Calbert, C.E., S. M. Greenberg, G. Kryder and H.J. Deuel,
        Jr. THE DIGESTIBILITY OF STEARYL ALCOHOL, ISOPROPYL
        CITRATES AND STEARYL CITRATES, AND THE EFFECT OF THESE
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        MARGARINE FAT. Food Res. 16(4), 294-305 (1951).

(4)   Deuel, H.J., Jr., S.M. Greenberg, C.E. Calbert, R. Baker,
        and H.R. Fisher. TOXICOLOGICAL STUDIES ON ISOPROPYL AND
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(5)   United Nations. World Health Organization. SPECIFICATIONS
        FOR THE IDENTITY AND PURITY OF FOOD ADDITIVES AND THEIR
        TOXICOLOGICAL EVALUATION: SOME EMULSIFIERS AND STABILIZERS
        AND CERTAIN OTHER SUBSTANCES. 10th Report of the Joint
        FAO/WHO EXPERT COMMITTEE On Food Additives. World Health
        Organ. Tech. Rept. Series 373 (1967).

(6)   World Health Organization. Seventeenth Report of the Joint
        FAO/WHO Expert Committee on Food Additives, TOXICOLOGICAL
        EVALUATION OF CERTAIN FOOD ADDITIVES WITH A REVIEW OF
        GENERAL PRINCIPLES AND OF SPECIFICATIONS: FAO/WHO/C/INF/73.3
        (1974).

(7)   United States. Code of Federal Regulations, Title 21,
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(8)   Luckmann, F.H., D. Melnick and J.D. Miller. A NEW ACCELERATED
        HOLDING TEST INVOLVING AERATION OF OILS IN IRON TUBES.
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(9)   Vahlteich, H.W., C.M. Gooding, C.F. Brown and D. Melnick.
        ESTERS OF CITRIC ACID IN STABILIZING EDIBLE OILS. Food
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(10)   Aktieselskobet Trombo. ANTITHROMBOSE PREPARATIONS. Neth.
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(11)   Hill, L.M., E.G. Hammond and R.G. Seals. EFFECT OF ANTI-
        OXIDANTS AND SYNERGISTS ON PEROXIDE DECOMPOSITION IN MILK
        FAT. J. Dairy Sci. 52 (12), 1914-16 (1969).

                    OTHER PERTINENT REFERENCES

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        STABILIZATION OF GLYCERIDIC OILS. U.S. Patent 2,699,395
        (Jan. 11, 1955).

(13)   Gooding, C.M., H.W. Vahlteich, and R.H. Neal (Best Foods,
        Inc.) SOYBEAN OIL COMPOSITION AND METHOD OF PREPARING
        SAME. U.S. Patent 2,485,633 (Oct. 25, 1949).

(14)   Gooding, C.M., H.W. Vahlteich, and R.H. Neal (Best Foods,
        Inc.). CITRIC ACID ESTERS. U.S. 2,518,678 (Aug. 15, 1950).

(15)   Gooding, C.M. (Best Foods, Inc.). SOYA BEAN OIL AND METHOD
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(16)   Gooding, C.M., H.W. Vahlteich, and C.F. Brown (Best Foods,
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(19)   Vahlteich, H.W., C.M. Gooding and R.H. Neal (Best Foods, Inc.).
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(29) Harris, B.R. FROZEN EGG PRODUCTS. U.S. Patent 2,902,372 (Sept. 1, 1959).

(30) Neal, R.H., H.W. Vahlteich and C.M. Gooding (Best Foods, Inc.). NUT MEATS. U.S. Patent 2,485,636 (Oct. 25, 1949).

(31) Neal, R.H., C.M. Gooding and H.W. Vahlteich (Best Foods, Inc.). POTATO PRODUCTS AND PROCESSES OF PREPARING SAME. U.S. Patent 2,485,635 (Oct. 25, 1949).

(32) Conrad, L.J. and H.S. Stiles (Kind & Knox Gelatin Co.). METHOD OF IMPROVING THE WHIPPING PROPERTIES OF GELATIN AND GELATIN-CONTAINING PRODUCTS AND THE RESULTING PRODUCTS. U.S. Patent 2,692,201 (Oct. 19, 1954).

EVALUATION OF THE TOXICITY

OF A NUMBER OF ANTIMICROBIALS

AND ANTIOXIDANTS

Sixth Report
of the Joint FAO/WHO Expert Committee
on Food Additives

Geneva, 5-12 June 1961

Published jointly by
FAO and WHO
and issued also as
WHO Technical Report Series, No 228

CONTENTS

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

ROME, 1962

finding that could be attributed to the diet. Loss of calcium or other fixed bases was not observed. The dental attrition was found to be slightly more marked than in the control groups.[3]

Biochemical aspects

In man, citric acid is an important intermediate in the *Krebs citric acid cycle*, which represents the pathway of aerobic oxidation of pyruvic acid in the body.

Comment on experimental studies reported

In evaluating the acceptance of citric acid, emphasis is placed on its well-established metabolic pathways. Toxicological studies on animals supplement this information.

Evaluation

Level causing no significant toxicological effect in the rat

1.2% (= 12 000 p.p.m.) in the diet, equivalent to 600 mg/kg body weight per day.

Estimate of acceptable daily intakes for man

	mg/kg body weight
Unconditional acceptance	0-60
Conditional acceptance	60-120

References

1. Gruber, C. M. & Halbeisen, W. A. (1948) *J. Pharmacol. exp. Ther.*, **94**, 65
2. Krop, S. & Gold, H. (1945) *J. Amer. pharm. Ass., sci. Ed.*, **34**, 86
3. Bonting, S. L. & Jansen, B. C. (1956) *Voeding*, **17**, 137

ISOPROPYL CITRATE MIXTURE

Definition	A mixture consisting of approximately 27% monoisopropyl citrate 9% diisopropyl citrate 2% triisopropyl citrate 62% mono and diglycerides
Description	More readily soluble in oils than citric acid.
Use	As a sequestering agent in antioxidant mixtures and in fatty foods.

Biological Data

Acute toxicity

Animal	Route	LD$_{50}$ (mg/kg body weight)	Reference
Rat	oral	2800-3700	1
Dog	oral	2250	1

Short-term studies

Rat

A group of rats were fed isopropyl mixture in the diet at the rate of 1500-2000 mg/rat per day for 6 weeks. There was no demonstrable effect on growth or mortality, and there were no pathological findings.[1]

Rabbit

A group of rabbits were fed isopropyl mixture at an average level of about 3600 mg/rabbit per day for 6 weeks. There was no demonstrable effect on growth or mortality and there were no abnormal findings post mortem.[1]

Dog

A group of dogs were fed isopropyl mixture in the diet at the level of 0.06% for 6 weeks with no demonstrable effect on growth or mortality and no pathological findings.[1]

Long-term studies

Rat

Groups of rats were fed isopropyl mixture in the diet at the levels of 0%, 0.28%, 0.56% and 2.8% for a period of two years. No deleterious effects were noted in any of the treated groups with respect to growth rate, mortality or histopathology of the tissues.[1]

Multigeneration studies likewise indicated that isopropyl mixture at a level of 2.8% in the diet was innocuous.[1]

Biochemical aspects

Studies in the rat showed that isopropyl mixture was readily absorbed when it was incorporated in the diet up to the 10% level.[2]

Comments on the experimental studies reported

No deleterious effects were observed in the short-term studies in rats, rabbits and dogs, or in the long-term studies in rats.

Evaluation

Level causing no significant toxicological effect in the rat

2.8% (= 28 000 p.p.m.) in the diet, equivalent to 1400 mg/kg body weight per day.

Estimate of acceptable daily intakes for man

	mg/kg body weight
Unconditional acceptance	0-7
Conditional acceptance	7-20

References

1. Deuel, H. J., Greenberg, S. M., Calbert, C. E., Baker, R. & Fisher H. R. (1951) Food Res., 16, 258

2. Calbert, C. E., Greenberg, S. M., Kryder, G. & Deuel, H. J. (1951) Food Res., 16, 294

SODIUM DIACETATE

Chemical name	Sodium hydrogen diacetate
Empirical formula	$C_4H_7O_4Na \cdot H_2O$
Structural formula	$CH_3COONa \cdot CH_3COOH \cdot H_2O$
Definition	Sodium diacetate is a molecular compound of sodium acetate and acetic acid, containing not less than 39% of free acetic acid.
Description	A white, hygroscopic crystalline solid with an acetic odour. 1 g is soluble in 1 ml of water.
Use	Mould and rope inhibitor in baked goods.

Biological Data

Acute toxicity

There is no direct information on the LD_{50} of sodium diacetate in animals. It is probably similar to that of neutralized acetic acid.

Animal	Route	Neutralized acetic acid LD_{50} (mg/kg body weight)	Reference
Mouse	oral	3310	1
Rat	oral	4960	1
Rat	oral	3530	2

March 11, 1974

Dr. R. K. Malik
Joint Secretary
Joint FAO/WHO
Expert Committee on Food Additives
Food Policy & Nutrition Commission
Food & Agricultural Organization
Via Delle Terme di Caracalla
00100 Rome
Italy

Dear Dr. Malik:

As a supplier of isopropyl citrate, we have been asked to comment on the proposal that was submitted to the Codex Committee on Food Additives. These comments have been incorporated in the attached proposed specification sheet. Isopropyl citrate refers to a mixture of isopropyl citrate esters (predominantly isopropyl citrate). For ease of use, the article of commerce consists of a blend of 38% of this mixture of esters in 62% of a blend of mono- and diglycerides.

We have reproduced the identification tests listed in the original specification. In our hands, the tests listed under "C" are much more effective than those listed under "B."

If you have any questions concerning this modified specification, please write to me.

Sincerely yours,

Edwin L. Sexton
Assistant to the Vice President
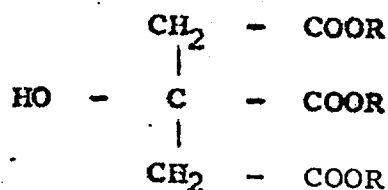Research and Quality Control

ELS:hg

cc: Dr. Durwood F. Dodgen
    Dr. Herbert Blumenthal
    Mr. Charles Feldberg

bcc: RJN
     CFB
     AEW

ISOPROPYL CITRATE

Chemical Name Mixed citric acid esters of 2-propanol

Structural Formula

$$
\begin{array}{ccc}
 & CH_2 & - \quad COOR \\
 & | & \\
HO \quad - & C & - \quad COOR \\
 & | & \\
 & CH_2 & - \quad COOR \\
\end{array}
$$

Where one R in the major component (Mono-isopropyl citrate) is isopropyl and the remainder is hydrogen.

DESCRIPTION

Chemical Description Approximate composition of isopropyl citrate:

Monoisopropyl citrate (major component) —
   25 parts by weight.
Diisopropyl citrate — 9 parts by weight.
Triisopropyl citrate — 4 parts by weight.

The article of commerce conforms to the following specifications: Isopropyl Citrate Mixture is composed of approximately 38 parts by weight of isopropyl citrate (distribution of esters indicated above) in 62 parts by weight of mono- and diglycerides and is an oil miscible semisolid material. The commercial product may be further specified as to saponification value, acid value, citric acid and isopropyl content.

Appearance Isopropyl citrate is a viscous, colorless syrup exhibiting some crystallization upon standing.

FUNCTIONAL USES Antioxidant, sequestrant.

CHARACTERISTICS

Identification Tests

A. Solubility

Water: soluble.
Ethanol: soluble.

B. Reflux 2 g of material with 50 ml
of sodium hydroxide TS for 1 hour.
Distill off 20 ml. Place 8 g chromic
oxide in a flask, add 15 ml water and
2 ml concentrated sulfuric acid.
Provide the flask with a reflux con-
denser and add 5 ml distillate slowly
through the condenser. Reflux for
30 minutes, then cool and distill
off 2 ml. Add 3 ml water and 10 ml
mercuric sulfate TS to the distillate.
Heat in boiling water bath for 3 minutes.
A white or yellow precipitate within
3 minutes indicates the presence of
isopropanol.

C. Reflux 3 g of material with 50 ml of
sodium hydroxide TS for 1 hour, and
stand to cool. This solution is used
for the following tests.

(i) Neutralize the solution with
diluted sulfuric acid (1 in 20),
add an excess of mercuric sulfate
TS, heat to boil, and add potassium
permanganate TS. The colour of
the solution disappears and a
white precipitate is produced.

(ii) Neutralize the solution with hydro-
chloric acid, add an excess of
calcium chloride TS and boil. White
crystalline precipitate which is
insoluble in sodium hydroxide TS,
but soluble in dilute hydrochloric
acid TS, is produced.

Purity Tests   Acids, other than citric acid, and
                      alcohols other than 2-propanol, should
                      be absent.

                      *Sulfated ash: Not more than 0.3%.

                      *Arsenic: Not more than 1 mg/kg.

                      *Lead: Not more than 10 mg/kg.

                      *Heavy metals: Not more than 30 mg/kg.

                      * See General Methods.

THE DIGESTIBILITY OF STEARYL ALCOHOL, ISOPROPYL CITRATES, AND STEARYL CITRATES, AND THE EFFECT OF THESE MATERIALS ON THE RATE AND DEGREE OF ABSORPTION OF MARGARINE FAT[a,b]

CLARENCE E. CALBERT, SAMUEL M. GREENBERG, GEORGE KRYDER, AND HARRY J. DEUEL, JR.

Department of Biochemistry and Nutrition, University of Southern California, School of Medicine, Los Angeles, California

(Manuscript received January 6, 1951)

The composition, functions, and non-toxicity of mixed isopropyl citrates, predominantly mono-isopropyl citrate (IC), and of the mixed stearyl citrates, predominantly distearyl citrate (SC), and of the alcohol components of these esters have been presented in an earlier publication (3). Mention was made of the limited solubility of IC in oils requiring a vehicle for effecting dispersion of the IC in the oils. For this purpose a mixture of mono- and diglycerides was employed. This solution of IC will be referred to hereafter as IC plus Vehicle. The detailed composition of these additives to oils for purposes of flavor stabilization has been presented in the earlier paper by Deuel, Greenberg, Calbert, Baker, and Fisher (3).

One purpose of the present study was to determine whether IC plus Vehicle or SC alters the rate of absorption of fat. Secondly, the digestibility of IC plus Vehicle and SC was also investigated. Finally, it was of importance to know whether either of the above components may exert any effect on the utilization of fat. In order to make a wider interpretation of the results possible, the digestibility studies were made on 2 species of animals, namely the rat and the dog. Tests were likewise carried out with a commercial stearyl alcohol to compare the utilization of this free alcohol with that of its citric acid esters.

EXPERIMENTAL

The studies on the effect of isopropyl and stearyl citrates on the rate of absorption of margarine fat were made by the procedure previously described (4). Such an experimental approach would test whether IC plus Vehicle or SC exerts an inhibiting effect on the lipolytic enzymes or on other absorption processes.

The procedures usually employed in this laboratory (2), modified as indicated below, were used for the digestibility tests. The experiments were made on 118 female rats, obtained from our stock colony, which weighed approximately 200 g. each. Ordinarily, 10 rats were used per group, although as few as 6 were employed in 2 cases, while one group consisted of 20 rats. The rats were placed on the diets containing the fats to be investigated for an orientation period of 5 days. Following this preliminary interval, the food consumption was recorded and the feces collected over a period of 8 days. The composition of the diets is recorded in Table 1.

The feces were analyzed for neutral fat and soap according to the procedures outlined by Augur, Rollman, and Deuel (1). Any unabsorbed IC, SC, or stearyl alcohol would appear with the neutral fat and/or soap fraction.

[1]

The digestibility of the total fat in the diet in the tests with IC plus Vehicle (Groups 1 and 2), and of the margarine fat when fed alone (Groups 8 to 11), is calculated by the usual method (1). In the case of the experiments on stearyl alcohol (Groups 3 and 4) and on SC (Groups 5 to 7), estimations of the digestibility of the stearyl alcohol or SC and of the margarine fat were calculated as indicated in the appendix.

The digestibility experiments on dogs were made over a 12-day period during the last third of the 12-week toxicological tests reported elsewhere (3). The feces were separated by the use of carmine markers which were fed on the first and thirteenth days of the test. The feces were well formed and could readily be collected quantitatively. The methods for analysis are largely similar to those employed in the rat tests.

TABLE 1

Diets used in the digestibility experiments on rats

Diet and group number	Percentage composition							
	Casein	Marga-rine fat	Sucrose	Salt mixture[e]	Yeast[d]	IC plus Vehicle	Stearyl alcohol	SC
1	18.0	22.5	46.0	7.0	4.0	2.5	......	......
2	18.0	15.0	46.0	7.0	4.0	10.0	......	......
3	18.0	23.2	46.0	7.0	4.0	......	1.8	......
4	18.0	17.5	46.0	7.0	4.0	......	7.5	......
5	18.0	22.5	46.0	7.0	4.0	......	......	2.5
6	18.0	15.0	46.0	7.0	4.0	......	......	10.0
7	18.0	24.87	49.0	4.0	4.0	......	......	0.13
8[c]	18.0	15.0	56.0	7.0	4.0[f]	......	......	......
9	18.0[g]	15.0	56.0	7.0	4.0	......	......	......
10	18.0[g]	22.5	48.5	7.0	4.0	......	......	......
11	18.0	25.0	49.0	4.0	4.0	......	......	......
12	18.0	0	74.0	4.0	4.0	......	......	......

[e] Osborne-Mendel salt mixture.
[d] Anheuser-Busch Strain G.
[c] Data from paper of Crockett and Deuel (2).
[f] One per cent liver extract (Wilson, 1:20) replaced an equal proportion of yeast.
[g] Vitamin-test casein used. Commercial casein was employed in all other tests.

In order to determine whether the stearyl citrates could be hydrolyzed by the dog, samples of fresh feces were removed from the cage in the period directly after the completion of the digestibility tests. These were stored in the deep freeze until analyses could be completed. An aliquot was removed for the determination of the dried stool percentage. Another aliquot was used for the determination of any citric acid in an aqueous extract. Citric acid was also determined in the aqueous extract following saponification of another aliquot while an analysis for stearyl alcohol was made on the N.S.F. of this sample. The content of stearyl citrates in the stool was calculated on the basis that the citric acid makes up the same proportion of the ester mixture as in the original SC preparation fed. Any stearyl alcohol remaining over and above that accounted for in ester form was considered to be present as the free alcohol. The method of Pucher, Wakeman, and Vickery (8) as modified by Perlman, Lardy, and Johnson (7) was used for the estimation of citric acid.

Table 2 gives the composition of the diets used in the digestibility tests with dogs.

The isopropyl and stearyl citrates were obtained from The Best Foods, Inc., while stearyl alcohol was a commercial product purchased from du Pont which bears the trade name stenol. The identities of these products were established by analysis in our laboratory. The data on the citric acid esters are reported elsewhere (3).

Stearyl alcohol was found to have the following constants: m.p., °C., 57 to 57.6 (59.4 to 59.8)[k]; and acid value, 0.0. Acetylated stearyl alcohol prepared from stenol gave the following values: saponification value, 177.6 (179.5, theory); m.p., 31 to 32°C. (32.85°C.)[k]; freezing point, 29.7°C. (30.25°C.)[k].

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[k] The values in parentheses (except that marked theory) are from Heilbron (6).

RESULTS

Experiments With Rats

Absorption Experiments. The absorption tests were made on male and female rats over 4- and 6-hour periods following the administration of margarine fat without or with IC plus Vehicle (3.33%) or SC (5%). The animals were adults which had been previously fasted for 48 hours. The margarine fat without or with the additives was fed at a level of approximately 300 mg./100 sq. cm. of surface area. A total of 91 rats was used in the study.

TABLE 2

Composition of the diets used in the digestibility tests with dogs

Food component	Composition of diet		
	D-1	D-2	D-3
	%	%	%
Commercial casein	20.0	20.0	20.0
Sucrose	35.0	35.0	35.0
Dextrin	12.2	12.2	12.2
Margarine fat[h]	18.3	18.24	15.3
Cod-liver oil (U.S.P.)	5.0	5.0	5.0
Cellu flour[i]	2.5	2.5	2.5
Salt mixture (10)	2.0	2.0	2.0
Dried brewer's yeast[j]	5.0	5.0	5.0
IC plus Vehicle	......	5.0	......
SC	......	0.06	3.0

[h] Without additives.
[i] Obtained from Chicago Dietetics Supply House.
[j] Anheuser-Busch, Strain G.

The rate of absorption was calculated by dividing the weight of the sample absorbed [weight of sample fed — weight of fat recovered from gut (corrected for residual content in fasting rat)] by the length of the experimental period. The correction for residual fat in the gastrointestinal tract of fasting male rats was found in these tests to be 59 mg. per rat while the figure obtained earlier for female rats which was used, was 30 mg. (1). These values were employed for correcting for residual fat in the gastrointestinal tracts of the experimental animals which would not be of dietary origin.

A summary of the results of absorption is included in Table 3.

Neither IC nor SC would appear to have any depressing effect in rats on the rate of absorption of margarine fat from the gastrointestinal tract at the levels given. The concentration of IC plus Vehicle used in these tests is 133 times the amount at which it is proposed to use this mixture (0.025%) in margarine fat, while SC was incorporated at somewhat more than 25 times the proposed maximum for usage (0.15% in margarine).

Digestibility Experiments in Rats. Table 4 records digestibility experiments in which IC plus Vehicle, stearyl alcohol, or SC was added to the dietary fat at several levels. In all cases, sufficient margarine fat was included to bring the total level of fat plus test substance to 25% of the diet. The control experiments are summarized in Table 5. These include tests in which margarine fat was fed at 15, 22.5, or 25% of the diet without the above additives (Groups 8 to 11). Group 12 gives the experimental

TABLE 3

The absorption of margarine fat without or with IC plus Vehicle or SC from the
gastrointestinal tracts of rats

Group no.	Category	Number of rats	Sex	Body weight after fasting	Fat Fed	Fat Absorbed Total	Fat Absorbed Per 100 sq. cm. per hour	Fat recovered
	Margarine fat alone or containing 3.33% IC plus Vehicle							
				g.	mg.	mg.	mg.	%
1	Recovery							
	a. Margarine fat alone	8	M	280	1083	......	......	91.7
	b. Margarine fat with IC plus Vehicle	8	M	264	1044	......	......	89.5
2	Absorption (4 hours)							
	c. Margarine fat alone	10	M	265	1045	593	41.7	......
	d. Margarine fat with IC plus Vehicle	9	M	259	1028	643	45.5	......
3	Absorption (6 hours)							
	e. Margarine fat alone	9	M	266	1046	780	36.5	......
	f. Margarine fat with IC plus Vehicle	9	M	283	1068	737	33.2	......
	Margarine fat alone or containing SC (5%)							
4	Absorption (4 hours)							
	g. Margarine fat alone	10	F	158	766	352	43.2	......
	h. Margarine fat with SC	8	F	158	797	345	43.5	......
5	Absorption (6 hours)							
	i. Margarine fat alone	10	F	161	774	569	36.0	......
	j. Margarine fat with SC	10	F	156	797	584	37.5	......

TABLE 4

Summary of digestibility experiments on rats of fats containing IC plus Vehicle
(Groups 1 and 2), stearyl alcohol (Groups 3 and 4), or SC (Groups 5 to 7)

Category	Group number						
	1	2	3	4	5	6	7
Number of rats used	9	9	10	9	9	10	9
IC plus Vehicle in diet, %	2.5	10.0	....	....	....	....	....
Stenol (stearyl alcohol) in diet, %	....	....	1.8	7.5	....	....	....
SC in diet, %	....	....	....	....	2.5	10.0	0.13
Increase in weight, g	4.1	5.2	3.2	−7.4	−2.0	0.0	−0.1
Eaten, fat, g	15.6	10.7	15.5	8.4	9.6	8.2	16.4
Eaten, test substance, g	1.74	7.16	1.20	3.58	1.07	5.45	0.086
Feces, dry weight, g	4.86	5.12	5.46	5.98	5.10	9.30	3.69
Feces fat, neutral fat, g	0.297	0.319	0.516	2.807	1.257	3.195	0.613
Feces fat, soaps, g	0.589	0.455	1.014	0.754	2.299	3.787	0.664
Feces fat, corrected N.S.F., g	....	....	0.131	1.632	0.902	3.419	0.032
Feces fat, total, g	0.886	0.774	1.530	3.561	3.556	6.982	1.277
Feces fat, corrected total,[1] g	0.638	0.515	1.254	3.258	3.357	6.734	1.035
Digestibility of fat alone[m]	96.2 ± 0.4	97.1 ± 0.3	91.9 ± 1.0	80.8 ± 2.1	77.1 ± 1.3	71.6 ± 3.0	94.1 ± 0.6
Digestibility of test substance[m]	....	....	88.6 ± 4.2	54.8 ± 5.7	5.7 ± 3.9	19.3 ± 4.8	54.3 ± 8.1

[1] Corrected for metabolic fat 50.5 mg. per g. dried feces. Group 7 is based on metabolic fat results of Group 12.

[m] Including the standard error of the mean calculated from the formula, $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$, where d is the deviation from the mean and n is the number of observations.

data of the tests for the determination of metabolic fat where a fat-free diet was employed. Ordinarily 10 rats were used per group although as few as 6 were employed in 2 cases, while one group consisted of 20 rats. All tests were made on female rats which weighed approximately 200 g. each since these were available at the time of the tests. A total of 115 animals was used in these tests.

TABLE 5

Summary of control digestibility experiments without fat or with
margarine fat at several levels

Category	Group number				
	8	9	10	11	12
Number of rats used	20	6	6	10	8
Fat in diet, %	15.0	15.0	22.5	25.0	0
Increase in weight, g	—1.0	2.8	2.5	—0.2	4.9
Fat eaten, g	10.7	10.2	13.6	15.0	0.0
Feces, dry weight, g	4.40	3.33	3.22	3.26	2.82
Feces fat, neutral fat, g	0.240	0.113	0.342	0.468	0.107
Feces fat, soaps, g	0.300	0.341	0.334	0.484	0.081
Feces fat, total N.S.F., g	0.091	0.058	0.192	0.116	......
Feces fat, total, g	0.540	0.474	0.678	0.952	0.188
Feces fat, corrected total, g.[n]	0.310	0.306	0.516	0.750	0.066[o]
Coefficient of digestibility[p]	97.0 ± 0.4	97.0 ± 0.9	96.3 ± 1.5	95.2 ± 0.9	......

[n] Corrected for metabolic fat of 50.5 mg. per g. dried feces. Groups 7 and 11 corrections are based on metabolic fat determined in Group 12.

[o] Total g. feces fat per g. dried feces (metabolic fat).

[p] Including the standard error of the mean calculated by the formula given in footnote [m], Table 4.

Experiments With Dogs

Digestibility experiments were made with 3 groups of 3 dogs each. One group comprised the controls in which the fat with no additives was fed, the second consisted of those in which the test diet included IC plus Vehicle (0.06% of the diet, 0.25% of the fat), while the final group received 3% SC in the diet (14.8% of the fat). The data of the individual tests are summarized in Table 6.

When the diet contained 18.3% margarine fat and 5% cod-liver oil without additives, the average coefficient of digestibility without correcting for the metabolic fat was 95.3. After correcting for the metabolic fat using the correction figure of 0.198 g./g. dried stool (5), the digestibility values for fat averaged 99.7%. The corresponding figures for digestibility of margarine fat containing IC plus Vehicle are 95.5% and 99.7% without and with the correction for metabolic fat.

On the other hand, considerably lower values were obtained for the digestibility of the lipid as a whole in the tests where SC was given. This average value was 79.8%. However, when correction is made for the SC and stearyl alcohol content of the feces, the coefficient of digestibility of fat (still uncorrected for metabolic fat) is approximately 87%. Finally, when the correction for metabolic fat is likewise applied, the mean coefficient of digestibility averages 94.5.

There are several assumptions that it has been necessary to make in order to calculate the digestibility of fat in these tests. In the first place,

TABLE 6
Digestibility tests of margarine fat and cod-liver oil in dogs when fed alone in the diet or with IC plus Vehicle or with SC

Category	Control tests			IC plus Vehicle tests			SC tests		
	Dog 1	Dog 2	Dog 3	Dog 5	Dog 6	Dog 7	Dog 9	Dog 10	Dog 11
Food eaten, g	980	980	3962	974	974	3325	968	968	2870
Fat eaten, g	228.3	228.3	923.1	226.9	226.9	775.0	196.5	196.5	582.6
Test substance eaten, g	......	......	......	0.58	0.58	2.00	29.1	29.1	86.1
Feces									
Dry weight, g	49.1	68.7	284.7	52.3	43.7	207.5	86.8	103.7	221.6
Corrected dry weight, g.^{a}	......	......	......	......	......	......	72.4	87.7	187.4
Lipid excreted									
Neutral fat, g	4.40	4.59	36.9	6.71	3.65	22.8	26.7	35.8	69.1
Soaps, g	3.60	3.35	29.3	2.70	3.05	26.2	10.5	12.1	30.1
Total, g	8.00	7.94	66.2	9.41	6.70	49.0	37.2	47.9	99.2
Corrected for metabolic fat, g.^{r}	0.00	0.00	9.2	0.00	0.00	8.0	......	......	......
Corrected for SC and stearyl alcohol only, g.^{a}	......	......	......	......	......	......	22.8	31.9	65.0
Corrected for SC, stearyl alcohol, and metabolic fat, g	......	......	......	......	......	......	8.5	14.5	27.8
Coefficients of digestibility									
Total lipid	96.5	96.7	92.8	95.7	97.1	93.8	81.1	75.6	82.8
Fat	100.0	100.0	99.2	100.0	100.0	99.0			
Corrected for metabolic fat	......	......	......	......	......	......	88.3	83.8	88.9
Corrected for SC and stearyl alcohol only	......	......	......	......	......	......	95.6	92.6	95.2
Corrected for SC, stearyl alcohol, and metabolic fat	......	......	......	......	......	......	50.5	45.0	60.2
SC alone									

^{a} Corrected by subtracting weight of SC plus stearyl alcohol (Item o, Table 7) from total weight of dried feces. ^{r} Corrected for metabolic fat by multiplying corrected dried feces weight by 0.198 (5).

since no figure for estimating metabolic fat has been reported for the dog, the same value has been assumed as for man. This figure would appear to be too high, since the metabolic fat calculated from it exceeds the figure for total fecal lipid found in 4 of the 6 tests on fat alone or fat with IC plus Vehicle. Moreover, since some of the citric acid from the stearyl citrate esters in the stool may be unavoidably destroyed on saponification, the resulting calculations for non-fat residues in the feces may be too low. This would cause the calculated coefficients of digestibility of fat to be too low. Such an error would have only a small effect on the results (less than 1%).

TABLE 7

The determination of the proportion of SC and stearyl alcohol excreted in the feces of dogs after the administration of SC

Category	Dog 9	Dog 10	Dog 11	Dog 12
Stearyl alcohol excreted:				
(a) N.S.F., total, mg.	258(1)	219(4)	......	230(2)
(b) N.S.F., control value, mg.	33	33	......	33
(c) Stearyl alcohol, total, mg. (a-b)	225	186	......	197
Citric acid excreted:				
(d) As free acid (before hydrolysis), mg.	0	0	......	0
(e) Combined (after hydrolysis), mg.	46(1)	49(4)	......	50(2)
Distribution of stearyl alcohol:				
(f) Total, mg. (c)	225	186	......	197
(g) Combined, mg. (calculated from e)*	129	138	......	140
(h) Free, mg. (f-g)	96	48	......	57
Calculation of SC hydrolyzed:				
(i) Total stearyl alcohol as SC, mg. (calculated from c)[t]	290	240	......	254
(j) Unhydrolyzed SC, mg. (calculated from e)[u]	166	178	......	181
(k) Hydrolyzed, mg. (i—j)	124	62	......	73
(l) Hydrolyzed, % (k/i × 100)	42.8	25.8	......	28.7
Calculation of SC and stearyl alcohol content of feces in digestibility tests (Table 6):				
(m) N.S.F., total, gm.[v]	14.7	16.1	34.8	......
(n) N.S.F., corrected, gm.[w]	12.3	13.2	28.6	......
(o) Stearyl alcohol and SC in feces, g. $\left[(n \times l) + (100-l) \times n \times \dfrac{696}{540}\right]$	14.4	16.0	34.2	......

Figures in parentheses give number of determinations of which preceding value is the average. Values a-k represent mg. per g. of dried stool.
* Combined citric acid × 2.81 (540/192).
t Corrected stearyl alcohol × 1.29 (696/540).
u Combined citric acid × 3.63 (696/192).
v Value analytically determined.
w Control N.S.F. (estimated as 17.2% of dried feces) subtracted from m. Item o determined as indicated from tentative value of n. Value obtained in item o subtracted from weight of dried feces in Table 6. Corrected dry weight × 33 mg. gives the final correction to be applied to item m to give item n. Final calculation was made of item o by procedure indicated and this figure was used for the several computations in Table 6.

However, if citrate is destroyed on saponification, a marked error in the amount of free stearyl alcohol calculated will result. This would give a figure somewhat too high for the digestibility of SC itself. Moreover, a further assumption has been made that the citrate obtained after hydrolysis is combined with stearyl alcohol in the same proportions as it is in SC. If it were exclusively combined as tristearyl citrate, then the percentage

[8]

of free stearyl alcohol calculated would be too high; if it were combined largely as monostearyl citrate, then the reverse would be true. Such variations would not change the corrections made for calculations of fat digestibility.

DISCUSSION

The experiments reported here with rats indicate that IC plus Vehicle is completely digested when administered in amounts as high as 10% of the total diet or 67% of the fat. There is no evidence that the inclusion of such high proportions of this product in the food results in a lowering of the coefficient of digestibility of the fat included in the diet. Thus, the average figures for digestibility of the fat as a whole were 96.2 and 97.1 respectively for the margarine fat in rations containing 2.5 or 10% of IC plus Vehicle. The values for the digestibility of margarine without IC plus Vehicle when fed at a 15% level were 97.0% (2 series of tests); when fed at 22.5 or 25% of the diet, the average figures for digestibility were 96.3 and 95.2% respectively. The almost complete digestibility of margarine fat containing IC plus Vehicle is in line with the results of the tests on the rate of absorption. No essential differences were found in this index, irrespective of whether IC plus Vehicle was added to the diet or whether margarine fat without additives was used.

The results on rats with IC plus Vehicle were likewise duplicated on dogs. Thus, a mixture of margarine fat and cod-liver oil without or with IC plus Vehicle gave the following coefficients of digestibility: No correction for metabolic fat, 95.3, 95.5; corrected for metabolic fat, 99.7, 99.7.

On the other hand, the addition of stearyl alcohol to the diet of rats to the extent of 1.8% (7.8% on the fat) significantly lowered the digestibility of margarine fat to 91.1%. When 7.5% of stearyl alcohol was included in the diet (43% on the fat), the coefficient of digestibility of the fat was lowered to about 81, while that of stearyl alcohol itself was only 55.

Our experiments indicate that SC is poorly absorbed by the rat. When SC was added to the diets at levels of 2.5 or 10% (11 or 67% respectively on the fat), the calculated digestibility of the ester was 5.7 and 19.3% respectively. A concomitant reduction in the digestibility of margarine fat from the usual high level of 96 to 97% to approximately 75 per cent was noted. On the other hand, when SC was added to the diet in an amount of 0.13% (0.5% on the fat), which is about 3 times the maximum amount required to protect the fat, there was no appreciable lowering in the coefficient of digestibility of the margarine fat included in the diet (94.1).

The depression in absorption of margarine fat as the result of the enormous amounts of stearyl alcohol or SC added to the fat is attributed in part to the influence that these additives have on the melting point of fat so supplemented. Thus, a mixture of 10 parts of SC and 15 parts of margarine fat has a melting point of 123°F. (Wiley) compared to 94°F. for the margarine fat alone.[x] It has been repeatedly demonstrated that high-melting fats are poorly digested (1, 2). Of interest in this same con-

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[x] Data supplied by F. H. Luckmann, The Best Foods, Inc.

nection is the fact that when SC was fed to rats at a lower level (5% of the fat) but still very much in excess of that proposed for the use of the esters in fats, no interference in the rate of absorption obtains.

When a comparatively high amount of SC was introduced into the diet of the dog (3% of the diet or 15% on the fat), the digestibility of the fat was 94.5% although the SC itself was digested only about 52%. The fact that SC exhibits less inhibition on the digestibility of margarine fat in the dog than in the rat may be an example of species difference.

The data indicate that stearyl alcohol is largely absorbed in the rat when present at 1.8% of the diet (coefficient of digestibility = 89). This is in harmony with the results of Stetten and Schoenheimer (9), who report a ready absorption of stearyl acetate when fed at a 1% level in the diet of normal rats. However, stearyl alcohol is less completely utilized when present in the diet at a 7.5% level (coefficient of digestibility = 55).

In view of the fact that stearyl alcohol is well absorbed at a 1.8% level, while SC is largely unabsorbed when fed at an equivalent concentration in the diet (2.5%), one is forced to postulate that the rat has a limited ability to hydrolyze this ester. On the other hand, there would seem to be ample evidence that the dog is able to split SC to a considerable extent. This conclusion is based on the fact that definite amounts of free stearyl alcohol have been demonstrated in the feces of the dog. Moreover, the calculated coefficients of digestibility of SC in the dog at a 3% level in the diet approximate 50, while in the rat the figure was 6 when the esters were present to the extent of 2.5% of the diet.

SUMMARY AND CONCLUSIONS

In an investigation of new additives for food usage, it is essential to supplement toxicological studies with biochemical investigations to determine the metabolic fate of the proposed food additives. This type of experiment is useful in explaining toxicological manifestations, if such should be noted.

Experiments have been carried out to determine the digestibility of stearyl alcohol, isopropyl citrates, and stearyl citrates, and the effect of these materials on the rate and degree of absorption of margarine fat. The latter is a typical food in which the citrate esters might be included. Incorporation of isopropyl citrate esters, predominantly the mono-isopropyl citrate in its mono- and diglyceride vehicle (IC plus Vehicle) in amounts as high as 10% of the diet results in a practically complete absorption of the product without lowering the digestibility of the dietary fat. Stearyl alcohol, included in this study for purposes of better interpretation of the experimental findings, is absorbed to the extent of 89% when given at a level of 1.8% of the diet. Under these conditions, practically no adverse effect on the digestibility of the fat given concomitantly is noted. However, when added to the diet to the extent of 7.5% (43% on the fat), stearyl alcohol is absorbed in the amount of 55% and the fat absorption is decreased to 81%.

Stearyl citrate esters, predominantly distearyl citrate (SC), are poorly absorbed by the rat when fed at 2.5 or 10% of the ration (11 or 67% respectively on the fat). The coefficient of digestibility of the fat fed with

[10]

SC is lowered from the usual 96 to 97.% to 75%. However, when SC comprises 5% of the fat, no interference in rate and degree of fat absorption occurs. It would appear that the incomplete digestion of SC by the rat is due to the inefficient hydrolysis of these esters in the gastrointestinal tract.

The dog is able to digest SC more effectively than is the rat. Thus, at a level of 3% in the ration (approximately 15% on the fat), fully 50% is digested, and no significant interference with the absorption of margarine fat occurs.

It may be concluded that IC plus Vehicle fed at a level more than 2500 times that proposed for use under practical conditions, or IC alone in an amount about 1000 times the maximal proposed level, will in no way interfere with the digestibility of fat. In the case of SC, there is no significant interference in the absorption of fat when the SC is present in an amount somewhat more than 25 times the maximal proposed concentration. The interefrence in fat absorption at higher levels is attributed in part to the increase in melting point of the fat plus additive blend, an artificial situation created in an attempt to determine levels at which interference might be noted.

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APPENDIX

Methods of Calculation of Digestibility in Tests with Stearyl Alcohol (Stenol), Groups 3 and 4, in Rats

A. Calculation of digestibility of total fat by usual methods *(1)*.

B. Calculation of digestibility of fat alone.
   a. Determination of total fat fed.
   b. Total fat excreted (corrected) = Total fecal lipids —
      [Metabolic fat[a] + N.S.F. (corrected)[b]].
   c. Digestibility of fat alone = $\dfrac{a - b}{a} \times 100$.

C. Calculation of digestibility of stenol.
   d. Determination of total stearyl alcohol fed.
   e. Stearyl alcohol excreted = Total N.S.F. excreted —
      Control N.S.F.[c] excreted.
   f. Digestibility of stearyl alcohol = $\dfrac{d - e}{d} \times 100$.

Methods of Calculation of Digestibility in Tests With SC (Groups 5, 6, and 7), in Rats

D. Calculation of digestibility of total fat by usual methods *(1)*.

E. Calculation of digestibility of fat alone.
   g. Determination of total fat fed.
   h. Total fat excreted (corrected) = Total fecal lipids —
   $$\left[\text{Metabolic fat}^a + \frac{\text{N.S.F.(corrected)}^b}{0.776^d} \right]$$
   i. Digestibility of fat alone = $\dfrac{g - h}{g} \times 100$.

F. Calculation of digestibility of SC.
   j. Determination of total SC fed.
   k. SC excreted = $\dfrac{\text{Total N.S.F. excreted — Control N.S.F. excreted}}{0.776^d}$.
   l. Digestibility of SC = $\dfrac{j - k}{j} \times 100$.

[a] Metabolic fat is determined by total lipid excretion (neutral fat plus soap) on fat-free diet.
[b] Non-saponifiable fraction (corrected) = Total fecal N.S.F. — N.S.F. of control groups receiving 15 or 22.5% of margarine fat alone (Groups 9 and 10).
[c] N.S.F. of control groups (Groups 9 and 10).
[d] Factor for conversion of stearyl alcohol to equivalent amount of SC.

[12]

TOXICOLOGICAL STUDIES ON ISOPROPYL AND STEARYL CITRATES[a,b]

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In view of the increased demand for margarine in recent years, a need has developed for new sources of edible vegetable oils for hydrogenation. Since the production of soybean oil has been markedly increased in the United States within the last decade, the margarine industry has naturally turned to this oil to supplement the supply of other vegetable oils. Thus, while 87 million pounds of soybean oil were used in 1941 for hydrogenation, out of a total of 257 million pounds of all vegetable oils, the quantity of this oil employed in a 12-month period, ending June 30, 1950, was reported as 233 million pounds out of a total of 701 million pounds used by the margarine industry. In other branches of the edible fat industries, there has also been an increased usage of soybean oil.

The chief difficulty in the use of soybean oil is its property of undergoing a so-called "flavor reversion." This change results in the development of an unpleasant taste. The reversion begins in both limpid and hydrogenated oils almost immediately after deodorization. According to Martin, Schepartz, and Daubert (20), Schepartz and Daubert (30), and Stapf and Daubert (32) this reaction is associated with the production of carbonyl compounds.

Citric acid is especially satisfactory as an anti-flavor-reversion agent. However, neither citric acid nor its salts dissolve in sufficient amounts in oil to be used conveniently for this purpose.

Vahlteich, Gooding, and Neal in rotating authorship (8, 9, 10, 11, 22, 23, 24, 35, 36, 37, 38, 39) have recently reported the synthesis and effectiveness of fat-soluble esters of citric acid in inhibiting the flavor-reversion of soybean oil, and in stabilizing the flavor of other fatty foods. The compounds were mixed mono-, di-, and tri-esters of citric acid, containing from 3 to 18 carbon atoms in the alcohol moiety. The mono-esters were the effective agents in stabilizing oil flavor while the di-esters, particularly the higher homologues, exhibited in addition emulsifying properties.

Before the above-mentioned esters of citric acid can enjoy any widespread application in foods, it is necessary to prove that they are not toxic.

Obviously, it is not possible to test in a thorough manner all of the esters of citric acid mentioned in the patents cited. The lowest and highest homologues in the series were subjected to exhaustive toxicological and metabolic studies. The lowest homologue selected was the mixture of

[1]

isopropyl citrate esters, predominantly the mono-ester, and the highest homologue selected was the mixture of stearyl citrate esters, predominantly the di-ester, having a melting point of 57 to 58°C. By the above it is not meant that the toxicity studies represent a blanket investigation of all homologues between the isopropyl and stearyl citrate esters. The basis of selection of the lowest homologue was because of its greatest effectiveness per unit weight in stabilizing the flavor of oils. The stearyl esters were chosen because these were far more readily soluble in oils than the lower members of the series. Indeed this same reasoning constitutes the bases for selecting these two types of esters in commercial application (25).

As far as the hydrolysis products of these selected esters of citric acid are concerned, there is no danger of toxicity, even for a consumption of many times the levels required to preserve the oils. Both hydrolysis products of stearyl citrate esters are components of animal tissues, and are produced by normal metabolic processes. Not only are citrates widely distributed components of foods such as the citrus fruits and milk, but they are also intermediary products of carbohydrate metabolism in the so-called citric acid cycle (14, 33). At the present time, the use of "citric acid and harmless citrates" is permitted in margarine without any specified limit, but only when used for the purpose of producing diacetyl (6).

Stearyl alcohol has been found by Schoenheimer and Hilgetag (31) to occur in the feces of dogs; cetyl alcohol, however, was found to be a normal constituent of the feces of dogs, cats, and men. In fact, Stetten and Schoenheimer (34) have demonstrated that an interconversion of stearyl alcohol and stearic acid is a usual metabolic process. Moreover, stearyl stearate has been shown by Hilditch (12) to account for 2.5% of the total lipid in the head oil of the sperm whale.

Although isopropyl alcohol has toxicological properties at relatively high levels, no effects of this compound have been demonstrated which do not also result from the ingestion of ethyl alcohol (16). Isopropanol has been shown to be approximately twice as toxic as is ethyl alcohol (18). However, doses from 0.75 to 5.28 ml. of isopropanol per kg. body weight per day have been administered to rats without the development of any untoward symptoms. Administration of this alcohol over prolonged periods failed to show evidence of any delayed toxic effects (16). It was likewise found that no deleterious effects on growth, pregnancy, or lactation resulted over several generations in rats given considerable quantities of isopropanol daily (19).

The isopropyl citrates are dispersible in fat with some difficulty whereas the stearyl citrates are readily soluble in fat. For this reason, the first type of citric acid ester is introduced into an oil in a suitable vehicle. For this purpose, a mixture of mono- and diglycerides (1:1) of a vegetable oil is most desirable (39).

The mixture of mono- and diglycerides which is used in conjunction with isopropyl citrate cannot be considered to be a substance possessing toxicity. Mono- and diglycerides occur physiologically in the digestion of fats (7). Alpha-monopalmitin has been identified as a minor constituent of adrenal lipids (29, 42), while Jones, Koch, Heath, and Munson (13) have recently reported the isolation of this monoglyceride from normal

[2]

hog pancreas. The satisfactory utilization of monoglycerides in feeding experiments has been reported by several workers. Ames, O'Grady, Embree, and Harris (1) have recorded that the growth and reproduction in rats fed diets containing 15 or 25% of monoglycerides, over 3 generations, were just as satisfactory as was the case when the corresponding amounts of cottonseed oil were employed. Mattson and Beck (21) also obtained as good growth in rats when mono- and diglycerides were incorporated in the diet as when corresponding amounts of neutral fats were used. These results are in line with unpublished results obtained in our laboratory. They also confirm the earlier experiments of Braun and Shrewsbury (2), who studied the nutritional value of monostearin. At the present time mono- and/or diglycerides of fat-forming fatty acids are permitted in margarine (6).

Although the experimental data cited above indicate that the hydrolysis products of isopropyl citrate and stearyl citrate esters, as well as mono- and diglycerides, are in most cases natural products and are non-toxic at relatively high doses, it is still necessary to prove that the unhydrolyzed compounds do not possess specific toxicities not revealed in studies of the individual components. For this reason, the experiments reported here on rats, rabbits, and dogs were carried out. No previous toxicological investigations have been reported on isopropyl or stearyl citrate esters.

The test compounds used are mixtures of mono-, di-, and tri-alkyl esters of citric acid. In Table 1 are listed the typical compositions of the compounds investigated. From the acid value and saponification value, it is possible to estimate the ratio of mono- to di- to tri-ester for each of the types under study. It will be noted that, in the case of isopropyl citrates, the mixture is predominantly mono-isopropyl citrate and that in the case of the stearyl citrates the mixture is predominantly distearyl citrate.

In the preparation furnishing the isopropyl citrate esters, there is about 62% of the mixture of mono- and diglycerides. Analyses conducted in our laboratory for the alcohol moieties in the isopropyl and stearyl citrate esters checked very closely with the calculated values confirming the composition of the esters as given in Table 1. Analyses for citric acid gave values somewhat below the calculated values.

It will be noted in the footnotes to Table 1 that there are some variations in the ratio of mono- to di- to tri-ester in each category. It is pertinent to point out that in the present toxicological studies and in the metabolic studies reported elsewhere (3) many different batches of the mixed esters were employed (12 different batches in the case of the isopropyl citrate preparation and 11 in the case of the stearyl citrates). Analysis of the individual batches indicated that all samples fell in the range of composition indicated in the footnotes to Table 1.

For the purpose of convenience in referring hereinafter to the test materials employed in this study, the isopropyl citrate esters, predominantly mono-isopropyl citrate will be referred to as IC. Similarly, the stearyl citrate esters, predominantly distearyl citrate, will be referred to as SC. When the isopropyl citrate ester, predominantly mono-isopropyl citrate, is fed in the mono- and diglyceride vehicle, it will be referred to as IC plus Vehicle.

[3]

TABLE 1

Typical Compositions of Compounds Investigated

Substance	Melting point, °C.	Acid value	Saponi- fication value	Composition	Typical analyses [c]		
					Iso- propyl alcohol [d]	Citric acid [e]	Stearyl alcohol [f]
					%	%	%
Isopropyl citrates in a vehicle con- sisting of mono- and diglycerides (1:1) of vege- table oil.	Liquid	160	370	27% mono- isopropyl citrate [g]	10.9	26.6	......
				9% di-isopropyl citrate	(11.0)	(30.0)	......
				2% tri-isopropyl citrate			
				62% mono- and diglycerides			
Stearyl citrates	57.8	88.6	248.1	12.5% mono- stearyl citrate [h]	......	25.5	77.9
				75% distearyl citrate	......	(28.3)	(76.8)
				12.5% tristearyl citrate			

[c] Figures in parentheses are calculated values.
[d] Isopropyl alcohol was determined after oxidation to acetone by refluxing with aqueous potas- sium dichromate and by precipitation with Deniges reagent (40).
[e] Citric acid was determined on the aqueous extract after saponification by the method of Pucher, Wakeman, and Vickery (28) as modified by Perlman, Lardy, and Johnson (27).
[f] Stearyl alcohol was determined as NSF by purification of the residue after saponification by filtration, drying, re-solution, and removal of the solvent.
[g] The 38% of isopropyl citrates is composed of about 65-80% mono-isopropyl citrates, 15-30% of di-isopropyl citrates and 5-10% tri-isopropyl citrate. Typical acid and saponification values are 410 and 690 respectively.
[h] The stearyl citrates are composed of about 10-15% monostearyl citrate, 70-80% distearyl citrates, and 10-15% tristearyl citrates.

The most important factors which determine the behavior of a sub- stance in the body are its digestibility and absorption. If a substance is non-absorbable and at the same time non-irritating to the gastrointestinal tract, there is little possibility that it will exhibit any toxic effects. Data on the absorption and digestibility of isopropyl citrates, stearyl alcohol, and stearyl citrates have been presented in another communication (3).

It was found that IC plus Vehicle in large amounts did not lower the high digestibility of margarine fat when fed to the rat or dog. On the other hand, when SC was fed at very high levels to the rat, the digestibility of the margarine fat was somewhat depressed. However, when the SC was given at a level of 3 times the maximal proposed level (25), assuming that all the fat in the diet was so treated, the digestibility of the margarine fat was not appreciably altered (3). In the case of the dog, no significant depression in digestibility of the margarine fat occurred when the level of SC in the fat was fully 80 times the maximal proposed level.

EXPERIMENTAL

In order to check for any possible toxicological effects of isopropyl citrate esters, predominantly mono-isopropyl citrate (IC), the same plus the mono- and diglyceride vehicle (IC plus Vehicle), and of stearyl citrate esters, predominantly distearyl citrate, (SC), tests have been carried out on rats, rabbits, and dogs. The use of the several species in such studies is in line with the suggestions of Lehman, Lang, Woodard, Draize, Fitzhugh, and Nelson (17) that at least 2 species (including one which is a non-rodent) be employed.

[4]

All the rats used in the several types of experiments were from our stock colony. LD₅₀ studies were conducted on IC, IC plus Vehicle, and on SC.

Acute tests were made on male and female rats at 3 levels for each test substance for a 6-week period. Control experiments in which no test substance was included in the diets were carried out in each series. In most cases, 10 rats of each sex were used per group.

The third type of test (chronic) employed with the rats was essentially a longevity experiment carried out over a period of 2 years with the same distribution in groups as in the acute tests. In both the acute and the chronic tests, the animals were sacrificed at the end of the experiment, and tissues were obtained for histopathological examination.

Another procedure used here for the deetection of any possible chronic or cumulative toxicity includes multigeneration studies. These involved raising the first generation of rats from weaning on the several test diets, and on a control diet, and continuing them on these regimens during the breeding and the lactation periods. When the young were weaned, the parents were discarded and the second generation was continued on the same diets which the parents had received. A similar routine was employed for succeeding generations through the weaning of the sixth generation, in the IC plus Vehicle tests, and of the fifth generation in the tests with SC.

In still another approach, additional groups of rats were used to ascertain whether these esters of citric acid in oils develop a toxicity when heated at 205°C. (a temperature used in deep frying) over a prolonged period (8 hrs.). The oils previously heated with or without the citric acid ester, as well as potato chips prepared by frying in such oils, were incorporated in diets which were then tested by growth studies on weanling rats over 11-week periods.

In the complete series of tests reported here (not including the absorption and digestibility studies), a total of 2398 rats was used.

Rabbits and dogs were employed for acute toxicity studies on the citric acid esters. The rabbit tests were continued for 6 weeks, while the experiments on dogs lasted for a 12-week period.[*] A histopathological examination was made of the tissues of all of the test animals at the conclusion of the experimental period. In addition, massive single doses of IC plus Vehicle or of SC were administered to dogs. A total of 57 rabbits and 24 dogs was used in the toxicity tests.

The LD₅₀ tests were carried out by the administration of the test substance by stomach tube to the fasted animals. In the rat tests, studies were made on IC, an equivalent isopropanol-citric acid mixture as control, IC plus Vehicle alone and in cottonseed oil, cottonseed oil alone as a control, and SC in cottonseed oil. The tests were considered positive if the animals died within periods of 4 to 6 days; this interval was chosen since, at that time, practically all the animals remaining alive were again gaining weight and in good nutritional condition. The dog experiments were carried out in essentially the same manner but because of necessity the number of test animals was restricted.

The diets used in the rat and dog experiments were made up of purified foodstuffs. The compositions of the various diets used are listed in Tables 2 and 3.

The rats used on the LD₅₀ tests had been maintained on our usual stock diet. They were fasted for 18 hours prior to the administration of the test substance by stomach tube.

Rabbits were fed throughout the tests on commercial rabbit pellets.[q] IC plus Vehicle was mixed with the rabbit pellets in amounts of 0, 1.9, 3.8, 7.5, or 15.0% (0.0, 0.72, 1.4, 2.8, or 5.7% IC, respectively). In the tests with SC, the pellets were mixed with 2 or with 11% of the melted substance. The diets fed the dogs in the 3-month acute tests are patterned after those described by Cowgill (4). Table 3 gives the composition of these diets.

The IC, IC plus Vehicle, and SC employed in the tests reported here were commercial products supplied by The Best Foods, Inc. The identity of the products was confirmed by our analysis. Hemoglobin determinations were made on the dogs by the acid hematin method.

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[*] Albers Rabbit Family Ration.

TABLE 2
The Composition of Diets Used in the Rat Tests

Isopropyl citrate esters, predominantly mono-isopropyl plus mono- and diglyceride vehicle (IC plus Vehicle)

Dietary component	Acute tests					Chronic tests			Multigeneration tests				Heated oil tests			
	70	80	81	82	83	84	85	86	71	72	87	88	73	89	74	90
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Sucrose	55.5	54.9	54.3	53.0	50.5	55.5	55.5	55.5	50.5	53.5	50.5	53.5	55.5	55.5	44.0	44.0
Whole margarine[i]	12.50	11.42	10.34	8.00	3.50	12.22	11.94	9.70	12.5	12.5	9.7	9.7	....	....	....	....
Margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	11.5[j]	11.44[j]	....	....
Fat-soluble vitamin mixture[k]	....	....	....	....	....	....	....	....	....	....	....	....	1.0	1.0	1.0	1.0
Yeast[l]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Salt mixture[m]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Liver powder[n]	....	....	....	....	....	....	....	....	5.0[o]	2.0[p]	5.0[o]	2.0[p]	....	....	....	....
Potato chips:																
Fried in margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0	....
Fried in margarine fat containing IC plus Vehicle (0.5%)	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0
IC plus Vehicle	....	1.68	3.36	7.00	14.00	0.28	0.58	2.80	....	....	2.8	2.8	....	0.06	....	....
IC content	....	0.64	1.3	2.7	5.3	0.11	0.21	1.1	....	....	1.1	1.1	....	0.02	....	....

Stearyl citrate, predominantly distearyl citrate (SC)

Dietary component	170	180	181	182	183	184	185	186	171		187	188	73	189	74	190
	%	%	%	%	%	%	%	%	%		%	%	%	%	%	%
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	19.0		19.0	19.0	20.0	20.0	20.0	20.0
Sucrose	55.5	54.2	53.0	50.5	45.5	55.0	53.5	45.5	51.8		51.0	43.3	55.5	55.5	44.0	44.0
Margarine fat	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.0		9.9	10.0	11.5[j]	10.64	....	....
Fat-soluble vitamin mixture[k]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0
Yeast[l]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	7.6		7.6	7.6	8.0	8.0	8.0	8.0
Salt mixture[m]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.8		4.8	4.8	4.0	4.0	4.0	4.0
Liver powder[n,o]	....	....	....	....	....	....	....	....	4.8		4.8	4.8	....	....	....	....
Potato chips:																
Fried in margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0	....
Fried in margarine fat containing SC (7.5%)	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0
SC	....	1.3	2.5	5.0	10.00	0.5	2.0	10.0	....	....	1.9	9.5	....	0.86	....	....

[i] 8 I U of vitamin D (as viosterol) added per g. food. [j] Heated for 8 hours at 205°C. [k] Composition was as follows: α-tocopherol, 0.5 g.; carotene (General Biochemicals, Inc.), 20 mg.; vitamin D_3 (crystalline Winthrop), 0.5 mg.; cottonseed oil to make a total weight of 100 g. [l] Anheuser-Busch, Strain G. [m] Osborne-Mendel salt mixture (26). [n] Wilson Laboratories. [o] 1:20 concentrate. [p] 1:50 concentrate.

TABLE 3

Composition of the Diets Used in the Experiments on Dogs

Food component	Composition of diet		
	D-1	D-2	D-3
	%	%	%
Commercial casein	20.0	20.0	20.0
Sucrose	35.0	35.0	35.0
Dextrin	12.2	12.2	12.2
Margarine oil[r]	18.3	18.24	15.3
Salt mixture (Reference 41)	2.0	2.0	2.0
Cellu flour[s]	2.5	2.5	2.5
Cod-liver oil (U.S.P.)	5.0	5.0	5.0
Dried brewers' yeast[t]	5.0	5.0	5.0
IC plus Vehicle	....	0.06	....
SC	....	....	3.0

[r] Without additives.   [s] Chicago Dietetics Supply House.   [t] Anheuser-Busch (Strain G).

RESULTS AND DISCUSSION

LD_{50} Studies

The acute toxicity, as revealed by the determination of the LD_{50} levels, has been ascertained for rats, and checked with dogs. The results of the tests on rats are summarized in Table 4.

The LD_{50} values of the several test materials as determined on rats are all quite high. The LD_{50} value with male rats for IC plus Vehicle, when given alone, exceeded 20.7 g./kg., while when it was administered in a ratio of one part of cottonseed oil to 2 parts of IC plus Vehicle, the value was greater than 17.2 g./kg. The corresponding figures for the female rats were 18.8 and 19.2 g./kg. This would indicate that supplementation of IC plus Vehicle with fat does not accentuate the LD_{50} figure.

When IC was administered in alcohol solution or in water, the toxicity was 2.8 to 3.7 g./kg. The slightly lower LD_{50} value for the females receiving IC in ethanol may be the reflection of the higher alcohol dose which was administered. In the tests with females where an aqueous solution was employed, the figure for LD_{50} approximated the value obtained for the males. The quantity of IC required to produce 50% mortality approximated that for its hydrolysis products. However, when IC is administered in a fat vehicle, more than double the amount of IC is required to produce a 50% mortality.

The LD_{50} level for SC exceeds 5.4 g./kg. in the case of both male and female rats. Since it was impossible to prepare a solution of SC, liquid at body temperature, in a higher concentration than 20%, the maximum dose (30 ml./kg.) which could be introduced into the stomachs of rats contained only 5.4 g./kg.

Twelve dogs were used to check the LD_{50} values obtained with rats. Massive single doses, viz. 12 g./kg. of IC plus Vehicle, 2.25 g./kg. of IC, and 5 g./kg. of SC, were not fatal to the dog. After a period of 2 days, the dogs were eating normally and showed no obvious ill effects from the dosage.

[7]

TABLE 4

LD50 Tests on Rats

Substance tested	Sex of rats	Data on different levels of tests						Estimated LD50[a]
IC plus Vehicle								*g./kg.*
Dosage (g./kg.)	M	2.1-20.7	......	......	......	......	......	>20.7
Number of rats		6	......	......	......	......	......	(>7.9)
Mortality (%)		0	......	......	......	......	......	......
Dosage (g./kg.)	F	2.7-15.5	17.7	21.0	23.6	27.0	......	18.8
Number of rats		4	10	10	11	10	......	(7.2)
Mortality (%)		0	30	90	89	100	......	......
IC plus Vehicle in cottonseed oil (2:1)								
Dosage (g./kg.)	M	0	11.1	14.1	16.1	17.2	......	>17.2
Number of rats		10	9	10	9	9	......	(>6.5)
Mortality (%)		0	11	20	22	33	......	......
Dosage (g./kg.)	F	0	12.0-13.4	15.8	17.9	19.2	22.2	19.2
Number of rats		10	20	10	10	10	10	(7.3)
Mortality (%)		0	0	20	10	50	50	......
IC								
75% in 10% ethanol								
Dosage (g./kg.)	M	2.1	2.7	3.3	3.9	......	......	3.7
Number of rats		10	10	10	10	......	......	......
Mortality (%)		0	20	10	70	......	......	......
15% in 10% ethanol								
Dosage (g./kg.)	F	2.1	2.7	3.3	3.9	......	......	2.8
Number of rats		10	10	10	10	......	......	......
Mortality (%)		10	40	90	100	......	......	......
75% in water								
Dosage (g./kg.)	F	2.25	3.0	3.75	4.5	5.25	5.25[v]	3.6
Number of rats		10	10	10	10	10	10	......
Mortality (%)		0	10	60	70	100	90	......
Isopropyl alcohol plus citric-acid in ethanol								
Dosage (g./kg.)	F	3.9[v]	3.9	5.25[v]	5.25[w]	......	......	4.5
Number of rats		10	9	10	10	......	......	......
Mortality (%)		0	22	90	100	......	......	......
SC (20% in cottonseed oil)								
Dosage (g./kg.)	M	0	0.9-1.8	2.7	3.6	4.5	5.4	>5.4
Sol. vol. (ml./kg.)		30	5-10	15	20	25	30	......
Number of rats		10	4	2	12	13	10	......
Mortality (%)		0	0	0	8	15	10	......
Number of rats	F	10	4	2	12	13	10	>5.4
Mortality (%)		0	0	0	0	0	0	......

a Figures in parentheses represent IC content of mixtures.   v Male rats.   w In water.

Short-Term (Acute) Tests

Experiments on Rats. Tests were made on IC plus Vehicle or SC when incorporated in the commercial margarine fat. Table 5 summarizes these data. No animals maintained on any of the diets died during the course of the tests.

There is no evidence, on the basis of the acute tests on adult rats, that either IC plus Vehicle or SC possesses any degree of toxicity. Thus, adult

TABLE 5

The Change in the Weight of Adult Rats Receiving Control Diets Alone or Similar Diets to Which Several Levels of IC plus Vehicle or SC Were Added

Diet no.	Test material in diet	Experiments on male rats						Experiments on female rats					
		Number	Body weight		Increase in weight	Total food eaten.	Total test substance eaten	Number	Body weight		Increase in weight	Total food eaten	Total test substance eaten
			Start	End					Start	End			
Isopropyl citrate esters, predominantly mono-isopropyl citrate, plus mono- and diglyceride vehicle (IC plus Vehicle)													
	%		g.	g.	g.	g.	g.		g.	g.	g.·	g.	g.
70	0.0	10	336	386	50.2±2.7	625	0	10	221	247	26.5±3.5	496	0
80	1.68(0.64)ˣ	10	326	364	38.3±5.3	568	9.54	10	208	230	21.8±4.6	447	7.51
81	3.36(1.8)	10	324	368	44.8±4.9	588	19.8	9	214	231	17.3±5.1	450	15.1
82	7.00(2.7)	9	331	373	42.0±6.7	597	41.8	10	210	226	15.5±3.5	437	30.6
83	14.00(5.3)	10	329	372	42.6±4.1	615	86.1	10	208	223	15.0±7.8	432	60.5
Stearyl citrate esters, predominantly distearyl citrate (SC)													
170	0	10	203	271	67.9±5.9	442	0	10	202	230	27.6±3.4	390	0
180	1.3	10	206	302	96.0±7.9	514	6.68	10	202	230	28.4±3.6	422	5.48
181	2.5	10	209	292	82.7±7.5	494	12.3	10	206	235	29.2±3.8	406	10.1
182	5.0	10	205	296	90.8±7.8	542	27.1	10	196	220	25.0±5.5	447	22.3
183	10.0	10	208	304	95.4±6.9	555	55.5	10	193	220	27.2±5.2	445	44.5

ˣ IC content of IC plus Vehicle in parentheses.

male rats receiving IC plus Vehicle at the highest level ingested 86 g. of this ester over a 6-week period (more than 2 g. per rat per day), and female rats ingested an average of 60 g. over the same period (1.42 g. per rat per day) without any demonstrable harmful effects. These correspond to a daily intake of 0.78 g. and 0.54 g. respectively of IC per rat per day in the 2 sexes.

The results of the acute tests on SC are quite similar to those on IC plus Vehicle. The average level of SC taken in by the male rats receiving the highest dosage was 55.5 g. (1.32 g. per rat per day) and that ingested by the females was 44.5 g. (1.06 g. per rat per day).

The slight average differences noted in the increases in body weight between the control animals and those receiving progressively increasing doses of IC plus Vehicle are not significant. These tests were conducted on adult rats of about 6 months of age and the growth responses show no parallelism with dosage. Likewise, no significance is attached to the apparently better growth responses of the adult animals receiving SC when compared to the responses of the control rats.

Experiments on Rabbits. Two types of experiments were carried out on rabbits receiving the IC plus Vehicle. In the first series of tests, the animals were fed on the basal diet (rabbit pellets) and the test substance was administered daily to the animals by stomach tube over the 6-week period. In the second and third series of experiments with IC plus Vehicle, and in all the tests with SC, the substances under examination were mixed with the crushed rabbit pellets before this material was fed to the rabbits. Table 6 shows the average results of these tests.

TABLE 6

The Change in Weight of Rabbits Receiving Control Diets or Those Containing Several Levels of IC plus Vehicle by Stomach Tube (Series I), or IC plus Vehicle or SC Mixed with Diet (Series II and III)

Group No.	Series	Test material in diet	Number	Body weight			Average gain in weight	Average total food eaten	Average test material given
				Start	3 weeks	6 weeks			
				IC plus Vehicle by stomach tube					
		%		g.	g.	g.	g.	g.	g.
1	I	0	2	2010	2204	2770	760	Ad libitum food intake not recorded.	0
2	I	2.2	3	2098	2407	2899	801		120
3	I	4.4	2	2300	2598	2885	585		240
4	I	9.2	1	2104	2380	2780	676		500
				IC plus Vehicle incorporated in diet					
5	III'	0	8	2503	2821	3263	760	5413	0
6	II	1.9	1	2092	2402	2735	641	5411	113
7	II	3.8	2	2026	2456	2985	959	5399	205
8	II	7.5	2	2550	2632	2902	352	5010	376
9	II	15.0	5	2166	2556	2927	761	5366	805
10	III'	11.3	8	2427	2952	3276	849	5175	585
11	III	22.5	8	2442	2702	3039	597	3803	856
				SC incorporated in diet					
12	III	2	8	2532	2729	3171	640	4763	95.3
13	III	10	8	2516	2599	2950	434	4428	442.8

' One rabbit died during course of test.

[10]

Only 2 deaths occurred among the 57 rabbits during the course of the test. One of these was in Group 5 (control) and the second was in Group 10 (receiving 11.3% of the composite, IC plus Vehicle). The average intake of IC plus Vehicle of 4 groups of rabbits (22 animals) exceeded 500 g. during the 6-week period. The maximum consumption for the period was by one rabbit in Group 11. The total amounted to 1045 g., or 24.9 g. daily; this approximates a daily intake of IC plus Vehicle of 1% of the body weight.

The intake of the SC was somewhat lower, averaging 443 g. for the group on the higher dosage. The highest consumption in this group was 513 g. of the test substance for the period, or 12.2 g. per rabbit per day.

The average gain in weight for the control rabbits was 760 g. for the 6-week period. Several groups receiving IC plus Vehicle (Groups 2, 7, 9, 10) equalled or exceeded this figure. In both groups receiving SC, the average gain in weight was somewhat less. All rabbits receiving SC showed excellent increase in weight, except one animal which lost weight during the early part of the test when it failed to eat; this rabbit began to regain its weight during the last week of the test. It had returned to approximately its starting weight at the termination of the experiment.

A histopathological examination was made on 311 sections of 284 organs from 24 rabbits by one of us (H.R.F.). These included sections of the liver, kidney, heart, brain, lung, spleen, stomach, small intestine, large intestine, pancreas, adrenal, and testicle of the 8 rabbits receiving the highest dose of IC plus Vehicle (22.5%), of the 8 animals given higher dose of SC (10%), and of the 8 controls (Group 5) in Series III. These were all males. There was no obvious morphological evidence of disease in the tissues of any group given diets in the categories listed.

Experiments on Dogs. Acute tests were made on 12 dogs over 11 to 12-week periods. Three groups of 4 each were used. The dietary regimens included a basal diet (control group), a similar diet including 0.06% IC plus Vehicle, and a third to which 3.0% of SC was added. The dogs used consisted of a litter of 6 cocker puppies, which were distributed equally between the groups, and 6 adult mongrels of unknown nutritional history. The adults were devocalized and dewormed before the test was started, while the puppies were devocalized during the second week of the tests. Because of the uniformity in origin and dietary background of the puppies, greater significance should be attached to weight changes of these animals.

All dogs received measured amounts of food once daily. In the case of the adult dogs, the amounts were first based on the quantities necessary to establish an optimum body weight, as calculated from Cowgill's formula (5). When this weight was attained, they were placed on a maintenance diet. In the case of the puppies, the diet was adjusted to give a steady rate of growth. Water was given ad libitum throughout. The data on these tests are included in Table 7.

There is no evidence of toxicity of IC plus Vehicle or SC when fed to dogs over a 12-week period. IC plus Vehicle was fed at a level of 0.25% of the fat, which is 10 times the level proposed for its use in margarine (0.02% of the whole margarine or 0.025% of the margarine fat). SC was fed at a level of 14.8% of the fat, which is 80 times the maximum level to

TABLE 7
The Change in Weight of Dogs Receiving a Control Diet or Diets Containing IC plus Vehicle or SC

Dog No.	Type of dog	Sex	Body weight				Gain in weight	Total food eaten	Total test substance eaten	Hemoglobin at end of test
			Start	After 4 weeks	After 8 weeks	After 12 weeks				
					Control group (Diet D-1)					
			kg.	kg.	kg.	kg.	kg.	g.	g.	%
1	Cocker pup	M	2.3	3.2	3.5	3.6	1.3	7990	....	12.9
2	Cocker pup	F	2.0	2.8	3.2	3.2	1.2	7927	....	14.2
3	Mongrel adult	M	15.9	18.2	18.5	18.8*	2.9	27230	....	15.9
4	Mongrel adult	M	13.5	15.4	15.9	16.3	2.8	16870	....	17.6
Average**			2.15	3.00	3.35	3.40	1.25	7958	....	....
				Group receiving IC plus Vehicle (Diet D-2)						
5	Cocker pup	F	2.3	3.3	3.7	3.8	1.5	7918	4.75	12.6
6	Cocker pup	F	2.5	3.1	3.6	3.8	1.3	7663	4.60	14.0
7	Mongrel adult	M	10.9	12.8	14.8	15.0*	4.1	23330	13.98	14.3
8	Mongrel adult	F	5.0	6.4	6.8	7.4	2.4	17290	10.37	18.6
Average**			2.40	3.20	3.65	3.80	1.40	7790	4.68	....
				Group receiving SC (Diet D-3)						
9	Cocker pup	F	2.1	3.2	3.7	3.8	1.7	7683	230.5	12.9
10	Cocker pup	F	1.8	2.6	3.2	3.3	1.5	7887	236.6	12.8
11	Cocker adult	M	12.7	15.5	17.1	17.3*	4.6	19320	579.6	13.6
12	Mongrel adult	M	5.2	4.8	5.0	5.7	0.5	7990	239.7	14.0
Average**			1.95	2.90	3.45	3.55	1.60	7785	233.6	....

* Weight at 11 weeks. ** Average of litter mate cocker puppies only.

be used in margarine (0.15% of the whole margarine or 0.19% of margarine fat).

The amount of food consumed in the 3 groups was comparable. Actually, in the case of the puppies, the gain in weight of the experimental groups somewhat exceeded that of the control group, although the former animals consumed somewhat less food. The same pattern was followed by the adult dogs. Hemoglobin levels determined before the dogs were sacrificed were practically identical for the several groups. A histopathological examination of the livers and kidneys of the dogs failed to reveal any evidence of pathological manifestations attributable to the test substance. Whatever divergencies from normal occurred were distributed equally among the several groups.

Long-Term (Chronic) Tests on Rats

Longevity Tests. In order to determine whether the materials tested may exhibit a toxicological action when their administration is continued over a prolonged period, chronic tests were carried out on rats for 2 years. The tests were discontinued at the end of this interval, so that the tissues could be obtained for pathological examination from animals which had remained alive until the termination of the experiment. Data on the body weights of the rats in the various series of tests at representative periods during the experiment, as well as on their survival, are included in Table 8.

With the exception of the control group in the distearyl citrate tests, 55% or more of the rats were still alive after receiving the various diets for a period of 2 years. When allowances are made for accidental deaths, the percentage survival in the tests with IC plus Vehicle were 59, 82, and 58% for the groups receiving the low, intermediate, or high level of test substance, with the value of 63% for the control group. In the experiments in which SC was added to the diets, the survivals amounted to 66, 65, and 55% for the groups receiving the progressively increasing proportions of the test compound, with an average of 44% for the corresponding control group.

No marked variations in weight between the experimental and the control groups were noted during the 2-year period. However, after 104 weeks, the average of the means of the weights of males and females still surviving in the IC plus Vehicle tests were as follows: Diet 70 (control), 414 g.; Diet 84 (0.28%), 400 g.; Diet 85 (0.56%), 425 g.; and Diet 86 (2.8%), 404 g. In the tests with SC, the corresponding average weights were: Diet 170 (control), 380 g.; Diet 184 (0.5%), 486 g.; Diet 185 (2.0%), 413 g.; and Diet 186 (10%), 388 g.

Histopathological sections of liver, kidney, heart, brain, lung, spleen, stomach, small intestine, large intestine, pancreas, adrenal, and testicle or ovary were examined. In the case of the rats receiving IC plus Vehicle at a 2.8% level, it is reported as follows: "It is my opinion that no specific pathological lesions developed which could be attributed to the product fed."[bb] An examination of the same tissues of a series of rats which had

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[bb] Report of Professor Ernest M. Hall, Department of Pathology, School of Medicine, University of Southern California.

TABLE 8

The Body Weights and Survivals of Rats Receiving Control Diets or Diets Containing Several Levels of IC plus Vehicle or SC

Category	IC plus Vehicle tests				SC tests			
	Diet 70	Diet 84	Diet 85	Diet 86	Diet 170	Diet 184	Diet 185	Diet 186
Test substance in diet, %	0	0.28	0.56	2.80	0	0.5	2.0	10.0
IC content	0	0.11	0.21	1.06	....	....	....	....
Male rats								
Number in group	10	10	10	10	10	9	10	10
Body weight:								
Start, g	35	36	36	36	35	33	34	32
After 32 weeks, g	339	362	346	324	361	361	338	328
After 64 weeks, g	421	471	452	419	423	475	423	423
After 96 weeks, g	488	546	507	467	471	597	488	474
After 104 weeks, g	520	495	543	461	454	610	524	482
Rats alive at end	7	5	5	4	3	4	6	5
Female rats								
Number in group	10	10	10	10	10	10	10	10
Body weight:								
Start, g	40	38	39	39	30	32	32	~28
After 32 weeks, g	228	230	216	222	220	222	208	217
After 64 weeks, g	287	268	263	255	267	282	272	259
After 96 weeks, g	308	296	316	328	297	359	294	309
After 104 weeks, g	309	304	307	348	305	363	302	294
Rats alive at end	5	5	9	7	5	8	7	6
Survival of all rats								
Uncorrected, %	60	50	70	55	40	60	65	55
Corrected for those killed accidentally, %	63	59	82	58	44	66	65	55

received distearyl citrate at a 10% level for 2 years was made by one of us (H.R.F.). Although most of the rats exhibited some diseased tissues characteristic of old age, no specific changes traceable to the diet could be noted. Metastatic calcification and tumor formation were noted in certain tissues examined; however, they are not considered to have special significance, inasmuch as they were noted in the tissues of both test and control rats.

Multigeneration Tests. The most sensitive method for the detection of dietary deficiencies or toxicity involves subjecting several generations of the animals to the test diets. All the factors of stress are brought to bear on the animal in such tests, namely growth, pregnancy, and lactation. Diets which are entirely satisfactory for maintenance, or even for growth, over a single generation may fail to be sufficient for reproduction; moreover, a dietary regimen which serves adequately for both growth and reproduction may be unsatisfactory for lactation. The latter stress offers the most severe test of the nutritional value of a diet.

In the multigeneration tests, weanling male and female rats (Generation 1) whose parents had been on the laboratory diet were raised on the test diets and were bred after 10 weeks (at 13 weeks of age). These rats were continued on the test diets during the subsequent pregnancy and lactation. Young from the first generation (Generation 2) were continued after weaning on the same test diets which their parents had received. At maturity the second-generation rats were bred and a routine similar to the above was repeated. In the case of the rats receiving IC plus Vehicle, the experiment was continued through the weaning of the sixth generation; in the tests with SC, the experiment was terminated when the fifth-generation rats were weaned. Table 9 summarizes the data on the growth of the successive generations, while Table 10 records the information to be gained from the pregnancy and lactation performances.

The multigeneration tests indicate that IC plus Vehicle is completely harmless when incorporated in the diet at a level of 2.8% (equivalent to 1.1% of IC). The rats remained fertile throughout the 5 generations. The total litters cast in the test group comprised 91% of the females bred (59 of 65); this figure was identical with that of the control group (90%, 65 of 72). The body weights of the progeny were likewise similar in the control and experimental groups. At the age of 3 days, the average weights were 7.6 and 7.5 g., while at the age of 21 days the mean values were found to be 34.5 and 35.1 g., respectively.

The results of the growth tests carried on from weaning for the successive 10-week period give added confirmation of the non-toxicity of IC plus Vehicle at the 2.8% level. The average 10-week weight of the fifth generation male and female rats on the test diet (254.7 g., 178.3 g.) not only exceeded the average of the first-generation rats on this test diet for the 10-week period (238.7 g., 176.6 g.) but it was also higher than that of the fifth generation of the control rats (223.4 g., 151.2 g.).

A similar analysis of the data indicates that SC exhibits no toxicity over 4 generations when fed at a level of 1.9% or 9.5% of the diet. The fertility remained high in all groups. The percentages of successful pregnancies were as follows: Diet 171 (control), 93% (55 of 59); Diet 187

TABLE 9

Body Weights of Successive Generations of Rats Receiving Control Diets or Those Containing IC plus Vehicle or SC

Diet No.	Test substance in diet	Generation	Male rats Number	Body weights Start	After 5 weeks	After 10 weeks	Female rats Number	Body weights Start	After 5 weeks	After 10 weeks
	%				*IC plus Vehicle tests*					
				g.	g.	g.		g.	g.	g.
71	0	1	10	30.1	158.6	246.9	15	30.5	123.6	175.3
		2	9	38.6	168.5	281.5	15	37.8	137.9	187.3
		3	10	36.5	151.1	243.0	16	34.5	113.2	157.1
		4	14	38.8	153.2	224.3	22	37.4	123.0	159.4
		5	13	37.5	143.8	223.4	26	35.9	113.8	151.2
87	2.8	1	11	30.4	155.2	238.7	15	30.3	133.0	176.6
		2	11	38.5	157.7	265.0	12	34.6	132.8	182.1
		3	17	35.0	134.7	227.5	16	34.5	121.2	174.7
		4	12	39.5	164.4	240.0	18	37.9	124.5	160.4
		5	17	38.7	178.1	254.7	21	37.6	136.3	178.3
					SC tests					
171	0	1	5	37.8	164.0	265.0	10	37.3	135.7	182.5
		2	14	34.0	156.6	243.6	21	35.7	125.2	166.3
		3	16	37.4	168.2	253.5	19	35.8	122.7	163.1
		4	18	36.9	161.7	289.3	22	36.5	126.1	179.0
187	1.9	1	5	38.2	167.4	273.0	10	37.3	132.8	177.5
		2	19	34.9	150.1	245.3	16	33.9	122.0	171.1
		3	14	37.1	175.2	257.8	20	35.5	131.7	172.9
		4	10	40.3	172.9	268.1	16	38.6	129.1	177.8
188	9.5	1	4	36.5	160.5	267.3	10	37.6	131.5	177.4
		2	19	36.6	153.7	246.0	26	32.5	125.3	165.4
		3	10	33.2	162.2	237.1	19	32.7	124.7	166.2
		4	13	43.8	174.9	273.3	19	39.5	132.0	178.8

TABLE 10

Pregnancy and Lactation Response of Successive Generations of Rats Receiving Control Diets or Diets Containing IC plus Vehicle or SC

Diet No.	Test substance added	Parents				Litter					
		Generation	Number of rats bred	Number of litters cast	Average time[cc]	Generation	Average number	Body weight		Total rats weaned	Died between 3rd and 21st day
								3 days	21days[dd]		
					days			*g.*	*g.*		
IC plus Vehicle tests										
71	% 0	1	15	13	31.0	2	8.2	7.6	38.3(49)	70	0
		2	10	10	43.2	3	6.2	7.3	28.1(14)	33	4
		3	10	10	35.4	4	6.9	8.0	38.6(28)	56	3
		4	22	17	34.9	5	6.4	7.7	37.0(21)	55	1
		5	25	25	36.1	6	5.8	7.3	30.4(28)	103+[ee]	1
87	2.8	1	12	12	28.8	2	9.9	7.5	35.8(77)	77	0
		2	8	8	31.4	3	8.3	6.9	34.5(35)	35	9
		3	7	7	38.2	4	6.7	7.7	35.2(14)	46	0
		4	17	17	29.6	5	7.9	8.2	34.3(91)	98	0
		5	21	15	42.2	6	6.3	7.2	35.9(28)	56+[ee]	0
SC tests										
171	0	1	10	9	28.0	2	9.4	6.9	34.0(35)	35	0
		2	14	13	27.3	3	8.3	6.6	34.3(35)	60	3
		3	19	17	27.7	4	7.7	7.2	33.6(35)	65	5
		4	16	16	26.6	5	7.1	6.9	34.5(63)	72	7
187	1.9	1	10	10	26.8	2	6.3	6.7	34.6(21)	41	0
		2	15	15	26.2	3	6.8	7.4	33.5(42)	58	0
		3	15	14	24.4	4	8.1	7.0	36.9(56)	83	3
		4	14	14	31.0	5	8.4	6.8	36.7(56)	71	2
188	9.5	1	11	11	30.2	2	7.6	7.0	34.5(43)	61	2
		2	23	23	26.2	3	8.5	7.2	35.5(105)	117	0
		3	16	14	26.5	4	8.5	8.5	41.0(56)	67	0
		4	14	14	27.4	5	9.4	7.6	37.1(84)	94	0

[cc] Period elapsing after the male and female rats were put in the same cages. [dd] Average weights of 7-litter rats only. Figures in parentheses give average number of rats. [ee] Experiment terminated before all rats were weaned.

(1.9% SC), 98% (53 of 54); and Diet 188 (9.5% SC), 97% (62 of 64). A 100% fertility was obtained in the fourth-generation rats from the control and both experimental groups. The body weights of the young were slightly higher in the experimental groups than in the control group; this would indicate that no interference with the lactation process had been occasioned by SC. The average weights of 3-day-old rats of all 4 generations were 6.9 g. for the control group, 7.0 g. for those receiving 1.9% SC, and 7.6 g. for those receiving 9.5% of SC in the diet. After 21 days, the comparable averages were 34.1, 35.4, and 37.0 g., respectively. Mortality from the 3rd to the 21st day was 15 for the control group, 5 for those which received 1.9%, and 2 for those which were fed 9.5% of SC.

In the 10-week growth tests, the averages of the mean weights of the 4 generations on the several dietary regimens showed little difference. The values for the male rats gave a grand average of 262.8, 261.1, and 255.9 g. for Diets 171, 187, and 188, respectively. In the case of the females, the corresponding grand averages were 172.7, 174.8, and 172.4 g., respectively. The mean weights of the rats after 10 weeks on both experimental diets containing SC were approximately identical, after 4 generations, with those of the first-generation animals on the same dietary regimen.

Growth Tests on Rats with Diets Containing Heated Fats Without or With Citric Acid Esters

Since it is known that some fats such as sardine oil become less completely digestible when subjected to a high temperature (15), it was considered desirable to make tests on margarine fat heated without or with IC plus Vehicle or SC to determine whether a change in nutritional value may occur. In order to establish whether such a decrease occurs, growth tests were made on weanling rats using fats so treated as well as potato chips prepared by deep-fat frying in such fats. If the growth responses of the heated fats containing either of the citric acid esters tested were found to be inferior to that of the control heated fat, such an effect might be ascribed either to decomposition products of the citric acid esters or to a decomposition of the fat caused by heating in the presence of the citric acid esters. Data on these tests are summarized in Table 11.

No adverse effects on growth were noted in either male or female rats over 10-week periods when margarine fats heated at 205°C. continuously for 8 hours and used for deep-fat frying were incorporated in the diets, as compared with control tests in which unheated margarine fat was employed in similar dietary regimens. Moreover, when IC plus Vehicle or SC was added to the test oils prior to the prolonged heating procedure, and these oils were then subjected to deep-fat frying for the 8-hour period, no toxicity developed. This fact was demonstrated by identical growth curves of rats receiving diets containing the fat heated with additives as compared with those of rats receiving diets containing the heated fat without additives as well as the unheated fat without additives.

In addition, no differences in the growth of weanling rats were observed when they were fed diets containing potato chips fried in margarine fats with additives over that noted when the potato chips had been prepared in margarine fat without these citric acid esters. These results indicate

TABLE 11

The Increase in Weight of Weanling Rats over 10-Week Periods Which Received Diets Containing Unheated Margarine Fat (Control), Margarine Fat Heated at 205°C. for 8 Hours Without or With IC plus Vehicle or SC, and Potatoes Fried in Such Fats

Diet No.	Category	Test material in diet [f]	Number of rats	Body weight Start	Body weight End	Gain in weight	Total food eaten	Total test substance eaten	Efficiency of diet [ff]
		Experiments on male rats							
		%		*g.*	*g.*	*g.*	*g.*	*g.*	
70	Unheated fat (Control)	0	10	35.4	258.2	222.8	819	0	6.68
73	Heated fat	0	10	36.2	265.5	229.3	758	0	7.08
89	Heated fat plus IC plus Vehicle	0.06	10	37.3	262.3	225.0	748	0.45	7.04
74	Potato chips from 73	0	10	39.9	260.4	221.4	758	0	7.43
90	Potato chips from 89	0.044	9	36.3	263.0	226.4	789	0.35	7.04
189	Heated fat plus SC	0.86	10	38.0	267.5	229.5	776	6.67	6.93
190	Potato chips from 189	0.68	11	39.0	265.1	226.1	793	6.40	6.68
		Experiments on female rats							
70	Unheated fat (Control)	0	10	39.7	180.2	140.5	701	0	4.93
73	Heated fat	0	10	38.1	187.0	148.9	670	0	5.20
89	Heated fat plus IC plus Vehicle	0.06	9	38.0	193.7	155.7	680	0.41	5.37
74	Potato chips from 73	0	9	37.2	182.1	144.9	677	0	5.42
90	Potato chips from 89	0.044	9	39.2	185.7	146.5	689	0.31	5.22
189	Heated fat plus SC	0.86	9	38.9	180.8	141.9	649	5.58	5.12
190	Potato chips from 189	0.68	8	39.4	181.3	141.9	699	5.65	5.00

[f] Based on content of citrate esters present in fat at start of heating. [ff] (G. gain in weight/calories consumed) × 100.

that the heating of IC plus Vehicle or SC in margarine oil for 8 hours at 205°C. does not produce toxic decomposition products nor does it cause the oil to be adversely affected.

Acknowledgment

SUMMARY AND CONCLUSIONS

The results of the present toxicological study of 2 new chemicals proposed for use as food additives may serve as an illustration of what constitutes adequate testing of new food chemicals. Mixtures of mono-, di-, and tri-alkyl esters of citric acid were evaluated for the safety of their use in stabilizing the flavor of oils and fats and foods containing such oils and fats.

Consideration was first given to an independent analysis on the part of the testing laboratory of the mixed esters of citric acid to check the claims made by the manufacturer that the products were actually what they were reputed to be. Because of the variations in composition of mixed esters, many different batches were employed in the current investigation; analyses were made of representative batches.

Consideration was then given to the possible hydrolytic products of the esters of citric acid, and evidence was cited from the literature showing that such hydrolytic products in themselves exhibit no toxicity. It was emphasized that the intact esters themselves must be subjected to exhaustive toxicological studies using 3 species, not more than 2 being rodents. LD_{50} values carefully determined on one species and checked with another, acute experiments on 3 species, longevity tests, multigeneration studies, and toxicological studies of the possible degradation products of the citric acid esters were all conducted. In addition, histopathological examinations of tissues obtained from sacrificed animals were made throughout the course of the investigation. When the major portion of the studies was completed, the data were presented to the Division of Pharmacology of the U. S. Food and Drug Administration for their criticism, and their suggestions were followed in carrying the toxicological investigation to completion.

More specifically, studies have been undertaken to determine whether 2 new flavor-stabilizing agents for fat products, namely isopropyl citrate esters, predominantly mono-isopropyl citrate (IC), the same plus mono- and diglycerides vehicle (IC plus Vehicle), and stearyl citrate esters, predominantly distearyl citrate (SC), possess any toxicity when measured by various appropriate indices.

The LD_{50} values for the 2 compounds studied were relatively high. That for IC plus Vehicle alone or dissolved in oil was approximately 19.0 g./kg. (7.2 g./kg. as IC), while the figure for SC exceeded 5.4 g./kg. in rats. No sex differences were noted.

There is no evidence from acute experiments on rats (6 weeks), rabbits (6 weeks), or dogs (11-12 weeks) of any toxicity as measured by growth, mortality, or pathological manifestations.

Longevity (chronic) tests were carried out on rats, for 2-year periods from weaning, on IC plus Vehicle at several levels (0.28, 0.56, and 2.80% of the diet) and on SC at 3 levels (0.5, 2.0, and 10.0% of the diet). No deleterious effects as compared with that of the controls were noted in any groups in so far as could be determined by growth rates, mortality, and histopathological examinations of tissues.

Another method for evaluation of possible chronic toxicity, namely multigeneration studies, likewise indicated that IC plus Vehicle, at a level of 2.8% of the diet, and SC, at levels of 1.9 and 9.5% of the diet, are completely innocuous. These conclusions are based upon fertility over 5 and 4 generations, lactation performance as judged by the 3-day and 21-day weights of the offspring, as well as by their survival for the nursing period, and by the rate of growth of rats of the several generations over a 10-week period following weaning.

Margarine fat containing 0.5% IC plus Vehicle or 7.5% of SC did not develop toxicity (as determined by rat feeding tests over 12 weeks) when subjected to deep-fat frying at 205°C. over an 8-hour period. Potato chips, fried in such fats, and which retained approximately 40% of the fat, were also entirely satisfactory for growth.

Histopathological examination of the tissues of the rats after 2 years, of rabbits after 6 weeks, and of dogs after 11 to 12 weeks on the test diets did not reveal any evidence of specific damage. In the case of rats and rabbits, the following tissues were subjected to histopathological examination: liver, kidney, gastrointestinal tract, spleen, heart, lung, pancreas, gonads, brain, and adrenals. Only the liver and kidneys were examined in the dog tests.

All tests for toxicity indicate that IC when given as IC plus Vehicle and SC are harmless at levels as high as 1.1 and 10% of the diet, respectively. The maximum level at which IC has been tested and proved to be harmless is more than 500 times that which would be ingested in margarine containing 0.02% of IC where this fat makes up 15% of the diet by weight. Similarly, the highest level at which SC has been tested and which has proved harmless is about 500 times greater than would be consumed when margarine containing 0.15% of SC makes up 15% of the diet by weight.

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This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization or of the Food and Agriculture Organization of the United Nations.

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SPECIFICATIONS FOR THE IDENTITY AND PURITY OF FOOD ADDITIVES AND THEIR TOXICOLOGICAL EVALUATION: SOME EMULSIFIERS AND STABILIZERS AND CERTAIN OTHER SUBSTANCES

Tenth Report
of the Joint FAO/WHO Expert Committee on Food Additives

Geneva, 11-18 October 1966

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1967

Annex 2

ACCEPTABLE DAILY INTAKES FOR MAN
OF SOME MISCELLANEOUS FOOD ADDITIVES

Compounds considered	Specifications available	Overall daily intake zone a (mg/kg body-weight)	
		Unconditional	Conditional
Ascorbic acid	Yes	0-2.5	2.5-7.5
Citric acid	Yes	Not limited	
Propyl gallate	Yes		
Octyl gallate	Yes	0-0.2 b	0.2-0.5 b
Dodecyl gallate	Yes		
Glucono-delta-lactone	Yes	0-15	15-50
Hexamethylenetetramine	Yes	Decision postponed	
Isopropyl citrate mixture	Yes	0-7	7-20
DL-Lactic acid	Yes		0-100 c
DL-Malic acid	Yes		0-100 d
D-Mannitol	Yes	0-50	50-150
Polyvinylpyrrolidone	Yes		0-1

a The first part of the overall acceptable daily intake zone is termed unconditional, and this represents levels that can be safely employed without further expert advice. The second part of the zone is termed conditional and represents levels that can be employed safely but at which it is thought desirable that some degree of expert supervision and advice should be readily available.

b As the sum of these gallates (calculated as gallic acid).

c Refers to content of D(—)-lactic acid.

d Refers to content of D(—)-malic acid. The maleic acid content of malic acid should not exceed 0.05%.

Annex 3

ACCEPTABLE DAILY INTAKES FOR MAN
AND CLASSIFICATION OF SOME FOOD COLOURS a

Compounds considered	Specifications available	Toxicological classification	Overall daily intake zone b (mg/kg body-weight)	
			Unconditional	Conditional
Beta-carotene	Yes	A		
Beta-apo-8'-carotenal	Yes	A	0-2.5 c	2.5-5.0 c
Methyl and ethyl esters of beta-apo-8'-carotenoic acid (C30)	Yes	A		
Canthaxanthine	Yes	A	0-12.5	12.5-2.5
Black 7984		CII		
Indanthrene Blue RS	Yes	CI		
Quinoline Yellow	Yes	CI		

a See the eighth report of the Joint FAO/WHO Expert Committee on Food Additives (1965), page 14.

b The first part of the overall acceptable daily intake zone is termed unconditional, and this represents levels that can be safely employed without further expert advice. The second part of the zone is termed conditional and represents levels that can be employed safely but at which it is thought desirable that some degree of expert supervision and advice should be readily available.

c As the sum of all these carotenoids.

**WORLD HEALTH ORGANIZATION
TECHNICAL REPORT SERIES** No. 539

Toxicological Evaluation of Certain Food Additives with a Review of General Principles and of Specifications

Seventeenth Report
of the Joint FAO/WHO Expert Committee
on Food Additives

GENEVA
1974

easily from the polysaccharide gums, which have a different chemical structure and a different use. A specification for the substance was prepared and will be published in due course. On the basis of the no-effect level in the rat, the ADI for man was estimated at 0–2.5 mg/kg body weight.

Isoascorbic acid and sodium salt

Adequate short and long-term studies have been carried out in the rat. The biochemical studies indicate that isoascorbic acid is readily metabolized and does not affect the urinary excretion of ascorbic acid. On the basis of the no-effect level in the rat, the ADI for man was estimated at 0–5 mg/kg body weight.

Isopropyl citrate mixture and monoisopropyl citrate

On the basis of the no-effect level in the rat, the ADI for man was estimated at 0–14 mg/kg body weight.

Stearyl citrate

The data for this compound were carefully re-evaluated and the previous ADI increased.

Thiopropionic acid and dilauryl ester

The previous conditional ADI was deleted. Because studies on the distearyl ester were insufficient for evaluation purposes, the ADI does not include this compound.

α-tocopherol and mixed tocopherols concentrate

On the basis of clinical experience with this vitamin, the ADI for man was estimated at 0–2 mg/kg body weight, which includes the previous unconditional and conditional figures.

Others

Other antioxidants considered include citric acid and its salts and nordihydroguaiaretic acid.

5.4 Emulsifiers and stabilizers

Ammonium salts of phosphatidic acids

Tentative specifications prepared at the thirteenth meeting of the Committee were changed into final specifications. An ADI was established.

Diacetyl tartaric and fatty acid esters of glycerol

For these substances, the previous unconditional and conditional ADIs were converted to an ADI of 0–50 mg/kg on the basis of the results of biochemical and metabolic studies and feeding tests in animals.

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Product	Tolerance	Limitations or restrictions
Monoisopropyl citrate	. . . .	
Potassium citrate	. . . .	
Sodium acid phosphate	. . . .	
Sodium citrate	. . . .	
Sodium diacetate	. . . .	
Sodium gluconate	. . . .	
Sodium hexametaphosphate	. . . .	
Sodium metaphosphate	. . . .	
Sodium phosphate (mono-, di-, tribasic)	. . . .	
Sodium potassium tartrate	. . . .	
Sodium pyrophosphate	. . . .	
Sodium pyrophosphate, tetra	. . . .	
Sodium tartrate	. . . .	
Sodium thiosulfate	0.1 percent	In salt
Sodium tripolyphosphate	. . . .	
Stearyl citrate	0.15 percent	
Tartaric acid	. . . .	

[¶ 55,508]   (7) Stabilizers

Product	Tolerance	Limitations or restrictions
Acacia (gum arabic)*	. . . .	
Agar-agar	. . . .	
Ammonium alginate *	. . . .	
Calcium alginate *	. . . .	
Carob bean gum (locust bean gum)	. . . .	
Chondrus extract (carrageenin)	. . . .	
Ghatti gum *	. . . .	
Guar gum	. . . .	
Potassium alginate *	. . . .	
Sodium alginate	. . . .	
Sterculia gum (karaya gum)*	. . . .	
Tragacanth (gum tragacanth)*	. . . .	

* Substances added from February 2 and August 4, 1960, proposed lists.

Product	Tolerance	Limitations, restrictions, or explanations
Trypotophane (L-and DL- forms)*	....	Food additive regulation § 121.1002.
Tyrosine (L- and Dl- forms)*	....	Food additive regulation § 121.1002.
Valine (L- and DL- forms)*	....	Food additive regulation § 121.1002.
Vitamin A	....	
Vitamin A acetate	....	
Vitamin A palmitate	....	
Vitamin B_{12}	....	
Vitamin D_2	....	
Vitamin D_3	....	
Zinc sulfate *	....	
Zinc gluconate	....	
Zinc chloride *	....	
Zinc oxide *	....	
Zinc stearate (prepared from stearic acid free from chickedema factor)*	....	

[*As amended, 38 F. R. 20036, effective January 23, 1974.*]

[¶ 55,507]   (6) Sequestrants [2]

Product	Tolerance	Limitations or restrictions
Calcium acetate	....	
Calcium chloride	....	
Calcium citrate	....	
Calcium diacetate	....	
Calcium gluconate	....	
Calcium hexametaphosphate	....	
Calcium phosphate, mono- basic	....	
Calcium phytate	....	
Citric acid	....	
Dipotassium phosphate	....	
Disodium phosphate	....	
Isopropyl citrate	0.02 percent	

[2] For the purpose of this list, no attempt has been made to designate those sequestrants that may also function as chemical preservatives.

* Substances added from February 2 and August 4, 1960, proposed lists.

¶ 55,507   21 CFR 121.101

A New Accelerated Holding Test Involving Aeration of Oils in Iron Tubes

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THE active oxygen method (A.O.M. test) described by King, Roschen, and Irwin (1) and modifications thereof by Riemenschneider, Turer, and Speck (2) as a means of evaluating the keeping qualities of oils and fats have been used by the vegetable oil and animal fat industries for many years. This method depends upon the aeration of oils and fats (usually freshly processed) in glass at 98°C. with the determination of the progressive increase in peroxide value as an index of oxidative deterioration. The time in hours required for the attainment of a certain peroxide value associated with an organoleptically detected rancidity, *viz.*, a peroxide value of 100 milliequivalents per kilogram of hydrogenated vegetable oil, is the value reported. In citing such data, the possibility of subsequent metal pickup changing the value is neglected. Oils and fats are transported from refiner to bulk users in sheet iron tank cars. Users cream the fat with other ingredients in sheet iron mixers or fry with these products in sheet iron vats. Then why conduct A.O.M. tests only in glass apparatus? Certainly, such tests on fats relatively free of metals can fail to predict which fat is better protected against subsequent metal pickup.

The object of the present report is to present an accelerated holding test for evaluating the resistance of oils and fats to oxidative deterioration catalyzed by prooxidant metals. By simply replacing the glass tubes in the conventional A.O.M. test with iron tubes of similar design, the factors of iron contamination and contact metal catalysis are now brought into play. The precision of the modified A.O.M. test is good when the iron tubes are properly prepared. The results on an absolute and relative basis obtained in testing fats by the conventional and modified A.O.M. stability tests show striking discrepancies.

Experimental

Test methods. The assembly for the A.O.M. test used in the current studies was that described by King, Roschen, and Irwin (1) except that the glass tubes in which the fats were aerated were now replaced by iron tubes made by welding a small iron plate to the bottom of a 7¾-in. length of ¾-in. iron pipe. An aqueous solution of potassium dichromate and sulfuric acid, each in 2% concentration (3) was employed for washing the incoming air in place of the potassium permanganate solution (1).

The preparation of the iron tubes prior to evaluation of the test fats has proved to be of primary importance, and the procedure to be described must be carefully followed in order to obtain reproducible results. The tubes are cleaned by placing them in a 1 + 1 solution of concentrated hydrochloric acid and water for three hours. The tubes are then scrubbed with a hard bristle brush in order to remove loose iron, rinsed well with water, followed by acetone and finally ether, and blown dry with nitrogen. The tubes are next rinsed twice with the liquid test fat. Twenty ml. of test fat are added to each tube for the test. In the event the test cannot be run immediately after the tubes have been prepared, the tubes are filled with the test oil and held at the melting point of the oil until ready for use. This oil is discarded, and the tube is rinsed twice with fresh portions of test oil and wiped with cheesecloth; and 20 ml. of the fresh oil are added to the tube for the accelerated test.

Approximately 2-gm. samples of the oils aerated at 98°C. were taken at periodic intervals for determination of the peroxide value and for flavor scoring. The peroxide values were determined on a 0.2- to 0.3-gm. sample by the method of Riemenschneider, Turer, and Speck (2) and calculated as milliequivalents per kilogram of fat. Acceptability of oil flavor as employed in this study means that the oils were still edible; the cooked flavor which develops during aeration of the oils at the elevated temperature in both glass and iron tubes was discounted.

For comparative purposes A.O.M. tests of the same fats, using the conventional glass tubes, were employed. In some cases iron as iron stearate was dissolved in the oils aerated in the glass tubes.

TABLE I

Precision of A.O.M. Test Conducted on Commercial Shortenings Aerated at 98°C. in Glass and Iron Tubes

Code	Identity[a]	Glass Tubes			Iron Tubes		
		(a)	(b)	Average	(a)	(b)	Average
		Hours to a 100 m.e./kg. peroxide value					
A	Lard plus 0.02% BHA, 0.01% PG and 0.005% CA	35	35	35	9	14	12
B	Lard plus 0.02% BHA and 0.005% CA	54	55	55	18	18	18
C	Hydrogenated vegetable oil	81	83	82	35	30	33
D	Hydrogenated vegetable oil	98	98	98	28	30	29
E	Hydrogenated vegetable oil	106	112	109	38	37	38
F	Hydrogenated vegetable oil	118	117	118	18	23	21
G	Hydrogenated vegetable oil	120	121	121	58	61	60
H	Hydrogenated vegetable oil	137	141	139	59	64	62
I	Hydrogenated vegetable oil	142	138	140	23	28	26
J	Hydrogenated vegetable oil	148	154	151	21	25	23
K	Hydrogenated vegetable oil	201	200	201	52	52	52
L	Hydrogenated vegetable oil plus 0.08% IC	208	216	212	174	178	176
		Reproducibility of the Values Obtained in Hours					
Based upon a single analysis		±2.7(1 S.D.)			±2.7(1 S.D.)		
Based upon the average of duplicate analyses		±1.9(1 S.D.)			±1.9(1 S.D.)		

a BHA = butylated hydroxyanisole; PG = propyl gallate; CA = citric acid; IC = isopropyl citrate esters, predominantly monoisopropyl citrate.

[1]

TABLE II

Precision of the A.O.M. Test Conducted on a Hydrogenated Vegetable Shortening Containing Various
Antioxidants and Aerated at 98°C. in Glass and Iron Tubes

Antioxidant	Glass Tubes			Iron Tubes		
	(a)	(b)	Average	(a)	(b)	Average
	Hours to a 100 m.e./kg. peroxide value					
None..	68	73	71	39	40	40
0.02% butylated hydroxyanisole..................................	136	136	136	44	41	43
0.005% citric acid..	136	136	136	61	56	59
0.006% propyl gallate..	112	116	114	44	47	46
0.10% lecithin..	133	138	136	33	33	33
0.08% isopropyl-citrate...	150	150	150	154	156	155
0.005% citric acid + 0.006% propyl gallate.....................	290	294	292	51	56	54
0.10% lecithin + 0.006% propyl gallate..........................	237	243	240	47	53	50
0.02% butylated hydroxyanisole + 0.006% propyl gallate + 0.005% citric acid...............	290	289	290	120	115	118
0.02% butylated hydroxyanisole + 0.006% propyl gallate + 0.08% isopropyl citrate.......	320	325	323	278	285	282
	Reproducibility of the Values Obtained in Hours					
Based upon a single analysis..	±2.7(1 S.D.)			±3.0(1 S.D.)		
Based upon the average of duplicate analyses...................	±1.9(1 S.D.)			±2.1(1 S.D.)		

Iron analyses were conducted on a number of oil samples by the A.O.A.C. orthophenanthroline method (4), following exhaustive hot acid extraction of a 20-gm. oil sample and using the concentrated acid extract as the test solution. The standard deviation, based upon recovery experiments, has shown that this method is accurate to within ±0.1 p.p.m. of iron.

Reproducibility of the conventional and modified A.O.M. tests. In Table I are listed values obtained in duplicate when commercial shortenings were aerated at 98°C. in glass or iron tubes. It will be noted that the reproducibility of values, expressed in hours, based upon a single analysis or one run in duplicate was the same in the tests conducted on the oils in glass or iron tubes. Actually, the precision of the results on the oils in the iron tubes was poorer in relation to the number of hours for completion of the test; in iron tubes the oils exhibited striking decreases in A.O.M. values. The only exception was Shortening L, the product containing 0.08% mixed isopropyl citrate esters predominantly monoisopropyl citrate (5). The lard samples (A and B) were poorly protected in the iron tubes despite fortification with antioxidants. The variability in results between different shortenings is attributed to variability in concentration of added but not declared metal sequestering agents; compare, for example, results obtained on Shortening G with those on Shortening J. It is very likely that Shortening G had been treated with citric acid at some stage in processing while Shortening J had not had the benefits of such treatment. But even in the case of Shortening G (or H for that matter), protection against the prooxidant effects of iron was not impressive. The striking results obtained with Shortening L support the conclusion that the limited solubility of citric acid in oils restricts its effectiveness under the method of test. Ease of use and ready solubility very definitely favor the esters of citric acid (5).

In order to check some assumptions made above on the likely presence or absence of metal sequestering agents in the commercial shortenings and in order to check further into the reproducibility of the modified A.O.M. test, a study was conducted on one shortening before and after supplementation with the more popular antioxidants alone and in combinations in the customary concentrations used commercially. The results of this study are summarized in Table II.

The reproducibility of values obtained by the conventional and the modified A.O.M. test in this series was comparable to that obtained with the different shortenings (see Table I). Statistical evaluation of the combined data in Tables I and II show that the values (averages of duplicates) by either the conventional or modified methods are reproducible to within ±2 hours, actually ±1.9 and ±2.0 hours, respectively, for one standard deviation. The standard deviation of a single value is 2.7 hours in the former and 2.8 hours in the latter test.

It will be noted from the results obtained with the hydrogenated vegetable oil in glass tubes that various antioxidant mixtures were apparently superior to the isopropyl citrate alone. This however was not the case when the tests were conducted in iron tubes. Under these circumstances the high concentration of isopropyl citrate conferred a degree of stability strikingly superior to that attained with the other antioxidants, alone and in combinations. The best antioxidant combination appeared to be butylated hydroxyanisole, propyl gallate, and isopropyl citrate. The latter replaced the small concentration of citric acid customarily used in antioxidant combinations.

Flavor scorings conducted periodically on the fats described in Tables I and II during progressive oxidative deterioration were in very good agreement with the corresponding peroxide values. It was established that the peroxide values accepted as end-points in the conventional A.O.M. test, *i.e.*, 100 milliequivalents per kilogram for hydrogenated vegetable oils and 40 for hydrogenated lard, were equally applicable to the oils aerated in the iron tubes. Since the curve of increasing peroxide value (or rancidity) was so steep for the lard products subsequent to the attainment of a 40 peroxide value, the reporting of all values obtained to the common end-point of 100 was preferred.

Significance of the modified A.O.M. test. It was anticipated in initiating the present study that the aeration of oils in iron tubes would provide a dynamic test system for evaluating the resistance of the oils to oxidation catalyzed by a prooxidant metal. Progressively increasing contamination of the aerated oil with iron in the modified A.O.M. test was expected. In practical operations an oil or shortening does not become contaminated with a specific quantity of iron or other prooxidant metal but exhibits a progressive pickup of such metals as the oil is progressively exposed to metal equipment in transportation, storage, and use. The addition of a specific quantity of iron, as iron stearate, to the oil in the conventional glass tube cannot simulate adequately what occurs under conditions of use.

In order to check the above assumption, a study was conducted to determine the rate of iron pickup in oils aerated in iron tubes and to compare peroxide development in such oils with that noted for the same oils supplemented at one time with known quantities of iron and then aerated in glass tubes. The influence of the mixed isopropyl citrate esters, predominantly monoisopropyl citrate (5), in stabilizing the aerated oils in both test systems was also evaluated.
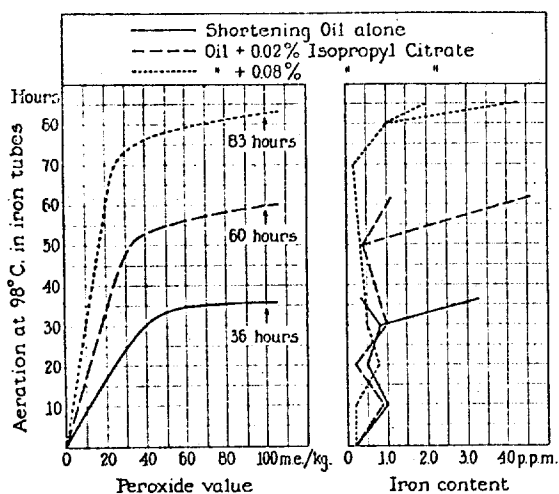

Fig. 1. Peroxide development and iron pickup during aeration in iron tubes of a hydrogenated vegetable oil with and without added isopropyl citrate esters, predominantly monoisopropyl citrate.

In Figure 1 are presented the results obtained in conducting A.O.M. tests on a hydrogenated vegetable oil aerated in iron tubes. This particular oil had an A.O.M. value of 79 hours according to the conventional test and 125 hours when protected with isopropyl citrate in either 0.02 or 0.08% concentration. The marked increase in the susceptibility of the oil to oxidation when aerated in the iron tube was again noted: 36 hours in contrast to the 79 hours obtained when the all-glass apparatus was employed. Isopropyl citrate protected the oil to such a degree that, at the 0.08% level, a value for the oil in the iron tube was obtained at least as good as that for the same oil without isopropyl citrate in the glass tube. This concentration of metal sequestering agent cannot be obtained with free citric acid.

Also plotted in Figure 1 are the iron values of the oils that had been aerated in the iron tubes. Because of the limited sensitivity of the colorimetric test for iron, each oil sample analyzed came from a different tube. This explains the erratic iron figures of from 0.2 p.p.m. to 1.0 p.p.m. as the oils were progressively aerated to the point of accelerated peroxide development. The variations in iron values were not due to imprecision of the test method. The method is accurate to within ±0.1 p.p.m. of iron, i.e., the standard deviation based upon recovery experiments. It was repeatedly observed during the periods of moderate increase in peroxide value, covering almost 90% of the aeration periods, that fairly good agreement between peroxide values of oils from duplicate tubes was obtained despite wide variations in the iron content of these oils. At the break point in the test, when the peroxide values sky-rocketed, there were marked

but still variable increases in iron content of the oils; the variability in the iron values is indicated in Figure 1 by the branching of the curves showing iron pickup. Indeed, when the oils were aerated for another hour or so, yielding peroxide values of 500 to 600, iron values as high as 30 p.p.m. were obtained. But here also there was poor correlation between peroxide value and iron content. The free fatty acid values paralleled to some degree the iron figures. During the major portion of the aeration period the free fatty acid values were small and variable from 0.06 to 0.20%, but at the point of sky-rocketing peroxide values the free fatty acid values were increased to about 1.3%. In the oils in the glass tubes the free fatty acid values increased by only 0.1%. The increase in free fatty acid values of the oils in the iron tubes at the terminal stages of the test may very well be responsible for the increased iron contamination.

In contrast to the results noted with the oils aerated in iron tubes are the findings in Figure 2 and
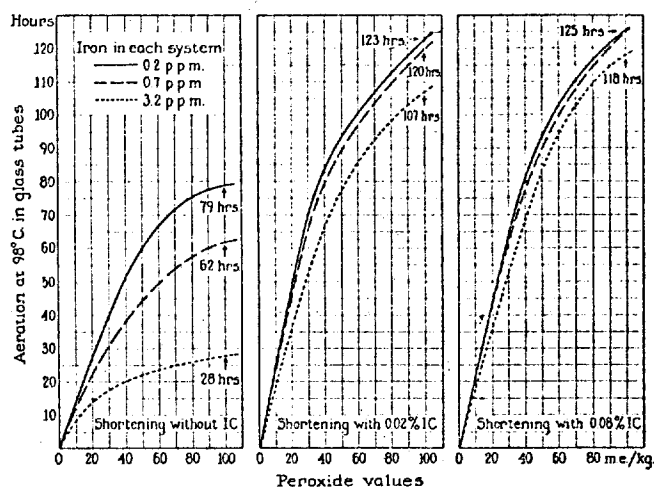
Fig. 2. Peroxide development during aeration in glass tubes of a hydrogenated vegetable oil with and without added isopropyl citrate (IC, predominantly monoisopropyl citrate) and before and after supplementation at one time with known quantities of iron as iron stearate.

obtained with the same oils aerated in glass tubes before and after supplementation at one time with known quantities of iron, as iron stearate. It will be noted that the acceleration in peroxide development at the terminal stages of the test was not as intense in glass tubes. The results on the oil without isopropyl citrate aerated in the iron tube (see Figure 1) most closely approximated the findings plotted in Figure 2 for the oil with 3.2 p.p.m. of iron, an iron value far in excess of that obtained during the major period of aeration of the unprotected oil in the iron tube. Isopropyl citrate at the two levels employed performed far more efficiently in protecting the oils against oxidation in glass tubes despite the far higher iron contents. Flavor scorings of the aerated oils in both glass and iron tubes confirmed the A.O.M. values presented.

It will be noted that peroxide values have been plotted on the abscissa and the hours aerated on the ordinate in both Figures 1 and 2. In so plotting the data, an index of overall keeping quality could be obtained. To evaluate the keeping quality by determining the number of hours to attain a specific

peroxide number (100 m.e./kg.) does not take into account the rate of peroxide development during the induction period. For a more critical evaluation the area under the curves presented in Figures 1 and 2 were measured. These values were expressed relative to the area obtained with the system "free" of iron set at 100; the basic oil in glass was rendered "free" of iron by the 0.08% isopropyl citrate. By this device, overall keeping quality of the oils was calculated.

TABLE III

Significance of A.O.M. Test Values on Shortenings With and Without Added Iron

Test system	Shortening with isopropyl citrate					
	0.0%	0.02%	0.08%	0.0%	0.02%	0.08%
Oil in glass tubes	Hours[a]			Overall quality[b]		
0.0 p.p.m. Fe[c]	125	125	125	100	100	100
0.2 p.p.m. Fe[d]	79	123	125	64	100	100
0.7 p.p.m. Fe[d]	62	120	125	48	95	98
3.2 p.p.m. Fe[d]	28	107	118	24	84	90
Oil in iron tubes	Hours[a]			Overall quality[b]		
Variable Fe content[e] of 0.2–1.0 p.p.m.	36	60	83	32	58	85
Fe content of oil in glass to give same A.O.M. picture for oil in iron tubes	p.p.m. of iron					
	2.5	Well above 3.2	Well above 3.2	2.0	Well above 3.2	More than 3.2

[a] To a peroxide value of 100 m.e./kg. of fat.
[b] Area under curve, plotting peroxide value versus hours of aeration at 98°C.; the area obtained with the system "free" of iron was set at 100 to obtain relative values for overall keeping quality.
[c] The basic oil in glass rendered "free" of iron by the 0.08% isopropyl citrate.
[d] Quantity of iron in the refined deodorized oil; the other values were obtained after supplementation with iron stearate.
[e] Just prior to the termination of the test.

The values for overall keeping quality have been listed in Table III. The hours required to attain the end-point in the A.O.M. tests are also presented for these test oils containing a variable iron content. It is apparent from the evaluation of results in Table III that the greater susceptibility of oils to oxidative deterioration in iron tubes is not due to the prooxidant effects of dissolved iron but primarily to contact metal catalysis and that isopropyl citrates minimize the deleterious effects due to both dissolved iron and contact metal catalysis. It would follow therefore that the present modification of the A.O.M. test is superior to the conventional A.O.M. test (even when the latter involves the addition to the oil of a specific amount of iron as a fatty acid salt) in evaluating the potential value of metal sequestering agents.

In Table IV are presented results obtained when another hydrogenated vegetable oil was aerated in iron tubes to a point just prior to sky-rocketing peroxide values. The oils were then transferred to glass tubes, and the A.O.M. test was carried to completion. From the results obtained it has been concluded that a marked instability has been imparted to the oils even during the early period of aeration in iron tubes and that isopropyl citrate is strikingly effective in protecting the oils during this period. It is postulated that the tocopherols (very weak acids) in the oils are either adsorbed by the metal wall and rendered ineffective or destroyed during the period of aeration. Thus the oil used in the A.O.M. test No. 6 may be regarded after the aeration in the iron tubes to be an essentially tocopherol-free oil. In those oils containing the isopropyl citrate esters, predominantly

TABLE IV

Instability Imparted to Shortening During Early Period of Aeration at 98°C. in Iron Tubes and Protective Influence of Isopropyl Citrate Esters (IC) in Minimizing This Effect

A.O.M. test	IC added	Aeration in tubes	Peroxide value attained	Hours Required	
				In each tube	Total
	per cent		m.e./kg.		
1	0.00	Iron	100	19	19
2	0.00	Glass	100	74	74
3	0.08	Iron	100	92	92
4	0.08	Glass	100	150	150
5	0.00	Iron and then glass	10 100	4 18	22
6	0.00	Iron and then glass	39 100	17 3	20
7	0.08	Iron and then glass	20 100	66 63	129
8	0.08	Iron and then glass	30 100	72 40	112

monoisopropyl citrate (a very much stronger acid), the competition for metal between tocopherols and monoisopropyl citrate is in favor of the latter. Under such circumstances the tocopherols should be better protected and therefore remain in the oil as effective antioxidants for longer periods of time. Studies dealing with the possible mechanisms involved in tocopherol retention are currently in progress.

It is recognized that the ratio of metal surface to fat in the iron tube test described in this paper is enormously greater than would be encountered in actual practice. However it should be remembered that the new test is an accelerated holding test designed to rate the effectiveness of antioxidants, particularly metal sequestering agents, in protecting oils. For an accelerated holding test the high ratio of metal to oil is not only desirable but required, just as high-temperature aeration of the oils in the conventional A.O.M. test is desirable and required for prompt evaluation of oils. The iron tube test undoubtedly overemphasizes the prooxidant effect of metals in promoting deterioration of oils in commercial operations; on the other hand, the conventional A.O.M. test fails to take into account the effects of dissolved metals and metal surfaces on oils under conditions of use. The relative keeping times of fats and oils in actual practice probably fall somewhere between these two extremes. Thus both tests have virtue in evaluating oils; the uniformity of values by the conventional A.O.M. test conducted on different batches of a given oil without antioxidants and metal sequestering agents reflects the reproducibility of the oil produced in the plant with respect to inherent resistance to oxidative deterioration; the values by the iron tube test reflect the degree of resistance of the oil to oxidation following extreme exposure to metal contamination and contact metal catalysis. Certainly, the iron tube test will predict which oil is better protected against the prooxidant effects of metals subsequently encountered.

Summary

A modification of the conventional A.O.M. test for evaluating the keeping qualities of fats and oils has been presented. This involves the substitution of an iron tube for the glass tube normally used in this test. The object in this change of procedure is to

permit evaluation of oils under conditions promoting oxidation catalyzed by metal pick-up and by contact with metal surfaces, situations encountered in commercial operations. It has been shown that a substantial lowering of the A.O.M. values is obtained when the modified test is used.

Data are presented on the reproducibility of the method for evaluating the stability of shortenings and the protective influence of antioxidants. Isopropyl citrate esters, predominantly monoisopropyl citrate, have proved to be superior to other metal sequestering agents in the test described since the esters can be readily added to oils in sufficiently high concentrations to be effective. Isopropyl citrate esters protect the oils not only against the prooxidant effects of dissolved iron but also against contact metal catalysis.

The relative keeping times of fats and oils in actual practice probably fall somewhere between the two extremes predicted from the results of the conventional and the modified A.O.M. tests. Both tests have virtue in evaluating oils; the reproducibility of values by the conventional test, conducted on different batches of a given oil without antioxidants and metal sequestering agents, reflects the reproducibil-

ity of the oil produced in the plant with respect to inherent resistance to oxidative deterioration; the values by the iron tube test reflect the degree of resistance of the oil to oxidation, following extreme exposure to metal contamination and contact metal catalysis.

All of the oils tested in the present study have been subjected to flavor evaluations in addition to serial determinations of peroxide value. The former have supported the latter relative to establishing the end-point or first sign when the oils develop rancidity.

Acknowledgment

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Esters of Citric Acid in Stabilizing Edible Oils[a]

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Uses of the esters of citric acid for stabilizing edible oils and fats are reviewed. The actions of these esters as metal deactivators and as inhibitors of flavor defects are demonstrated. A modification of the A.O.M. stability test is presented as a more critical evaluation method for oils and antioxidants.

Vegetable oils are generally rich in natural antioxidants, chiefly the tocopherols, which are phenolic-type compounds. They are therefore more resistant to oxidation than the animal fats, which are practically devoid of natural antioxidants. This explains the practice of supplementing animal fats, such as lard, with phenolic-type antioxidants, e.g., propyl gallate (13) and butylated hydroxy-anisole (6). Commercially processed oils and fats contain unavoidable trace metal contaminants, principally iron and copper. It is impractical and impossible to prevent the pick-up of these trace metals in the refining of oils and fats. In fact, the original seed or bean will contribute metals in amounts which in themselves are deleterious to the oil (4). The practical solution to the problem of trace metals is the use of an edible organic acid, or its derivative, as a metal-deactivator.

One practice today is to add citric acid to the oils undergoing processing (2). This method for stabilizing oils has several disadvantages. In the first place, a maximum of only about 0.005% citric acid can be dissolved in the oil. Also, heating of oils at the very high temperatures required for the rapid, though markedly limited, solution of free citric acid, viz., in the deodorization process, admittedly gives rise to a large number of different degradation products of known and unknown composition (3, 8). Operational difficulties, such as plugging of valves with solid citric acid, may also be encountered. The concentration of free citric acid in such treated oils is therefore variable and unknown. Use of free citric acid for stabilizing an oil or fat permits no control over the quantity of agent present in the supplemented oil, unless it is added in exceedingly small, suboptimal quantities following deodorization.

In this laboratory, we have discovered that a particular class of citric acid esters may be readily added to oils without any of the disadvantages cited above. More particularly, the solution to this problem involved supplementation of oils, following deodorization, with one of the mono-esters of citric acid (14).

Esters of citric acid as metal deactivators. During the course of these investigations, we were able to demonstrate that the esters of citric acid to be effective as metal deactivators must have 2 free carboxyl groups. Di- and tri-esters are completely inactive in this respect. The isopropyl citrate esters, predominantly monoisopropyl citrate, in a mono- and di-glyceride vehicle are particularly well adapted to the treatment of edible oils immediately following deodorization and preferably before

exposure of the oils to air. The higher homologues, such as stearyl citrate, are soluble in oils and require no vehicle. Both the isopropyl (1, 5, 10) and stearyl citrate (5) esters have been approved by Federal agencies for stabilizing edible oils and fats. In so far as degree of effectiveness of their monoesters is concerned, the isopropyl and stearyl citrates are equivalent mol. for mol. They blanket the practical range of citrate esters from an economic standpoint.

In Figure 1, the individual and combined actions of the true antioxidant of the phenolic type and of the metal deactivator are
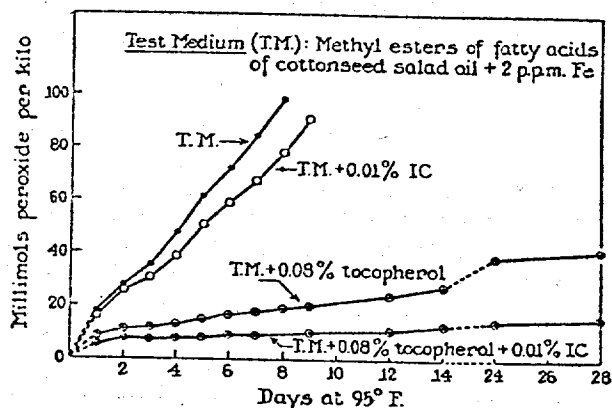

Figure 1. Individual and combined actions of phenolic-type antioxidants and of metal-deactivators in protecting fatty acid esters against atmospheric oxidation. IC indicates isopropyl citrate esters.

demonstrated by means of a special test system. The test medium consists of specially prepared[b] methyl esters of cottonseed salad oil fatty acids to which has been added 2 p.p.m. of iron in the form of iron stearate. Such a medium is the equivalent of cottonseed salad oil free of antioxidants but containing metal pro-oxidants. One-hundred g. samples in pint-size mayonnaise jars were exposed to air at 95° F. At intervals, peroxide value determinations were made according to the method of Wheeler (15). The metal-deactivating effect of mono isopropyl citrate is noted in the protection of the medium against oxidation. When tocopherol is added to the test medium, a marked stabilization against peroxide formation occurs; then, in the presence of the metal-deactivator plus tocopherol, a much greater re-

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[b] The esters were prepared by alkaline-catalyzed methanolysis of cottonseed salad oil, which had been previously bleached with 5% Norit (American Norit Co., Inc., New York, N. Y.), followed by a vacuum-distillation. The distilled esters were then diluted with an equal volume of mixed hexanes (Skellysolve-B, Skelly Oil Co., Kansas City, Mo.), and the solution passed through a column made up of a mixture of equal weights of Norit and Johns-Manville Hyflo Supercel. The solvent was removed by means of a vacuum-stripping and the esters were once again vacuum-distilled. This distillate was then subjected to acetylation employing a reaction mixture of 500 g. of esters, 100 ml. of acetic anhydride, and 3 drops of concentrated sulfuric acid as catalyst, in order to inactivate any residual tocopherol. The reaction product was washed thoroughly with hot water and after drying was subjected to two successive vacuum-distillations. The final distillate was stored at 25° F. overnight and used the next morning in the test discussed above. This obviously overcautious procedure was used in an attempt to obtain a tocopherol-free substrate. The Rawlings modification (11, 12) of the Emmerie-Engel method showed no tocopherols in the esters prepared in this manner.

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sistance to oxidation is attained, as revealed by an examination of the slopes of the respective curves. The monoesters of citric acid exhibited no activity in protecting the distilled methyl esters free of trace metals and polyphenolic antioxidants; the same effect has been noted with free citric acid in comparable test systems (7).

In Figure 2 is illustrated an application of the pure monoesters of citric acid in the stabilization of commercial salad oils against oxidation. The test and analyses were run in the same manner as described above in connection with Figure 1. Both these oils contained by analysis (11, 12) natural tocopherols in about 0.08% concentration. In the upper half of this figure the lack of function on the part of the distearyl ester is apparent, while the mono-ester is markedly effective in protecting corn salad oil from peroxide formation. The striking differences in the stability of soybean salad oil with and without a monoisopropyl citrate supplement are pictured in the lower portion of Figure 2. An extensive program based on studies such as these demonstrated that commercially refined and deodorized oils containing unavoidable trace metal contaminants can be readily stabilized by means of the mono-esters of citric acid.
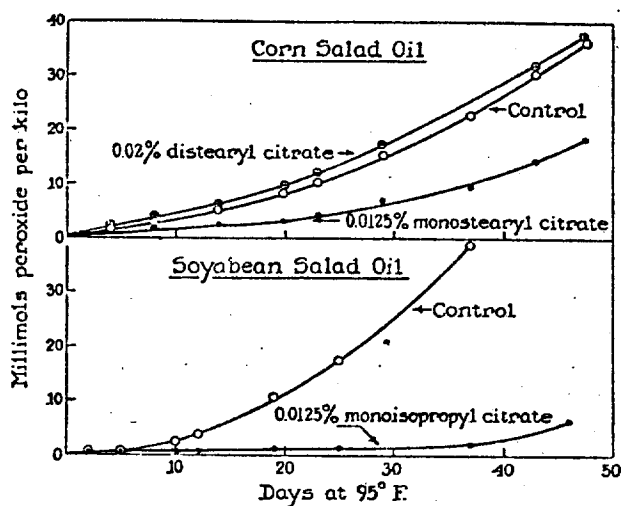
Figure 2. Protection of commercial salad oils against atmospheric oxidation following supplementation with pure citric acid esters. Oils contain approximately 0.08% native tocopherols.

Flavor tests using an expert panel. Evaluation of the effectiveness of the ester preparations for uses on a practical scale has not been based solely on peroxide value determinations. Repeatedly, various oil samples under code were subjected in our laboratory to flavor scorings by an expert panel in order to evaluate the stabilizing influence of the esters in protecting the flavor life of edible oils. For these tests covering oils stabilized according to current commercial practices, the readily available mixed citric acid esters were employed. The isopropyl citrate esters were predominantly monoisopropyl citrate, while the stearyl citrate esters were predominantly the diester, still containing an effective concentration of monostearyl citrate.

As an illustration of the effectiveness of the commercially available citrate ester preparations, the results of one objective flavor scoring study are presented in Figure 3. All samples were refined deodorized oils derived from the same starting refined soybean oil: The first sample was a soybean salad oil; the second, the same soybean oil hydrogenated for margarine manufacture; and the third, the same soybean oil hydrogenated for shortening manufacture. Half-filled quart-size mayonnaise jars containing the samples were stored under air at 95° F. The oils were scored at 2-week intervals by a panel consisting of 8 people well experienced in evaluating oil flavors. At scoring times, the samples were removed from the 95° F. room, melted where necessary and 5-g. portions for each panel member were poured into small brown bottles. This procedure permitted the careful warming of each flavor sample as the panel member was available for scoring without repeated meltings of the large storage sample. The average numerical scores reported by the panel
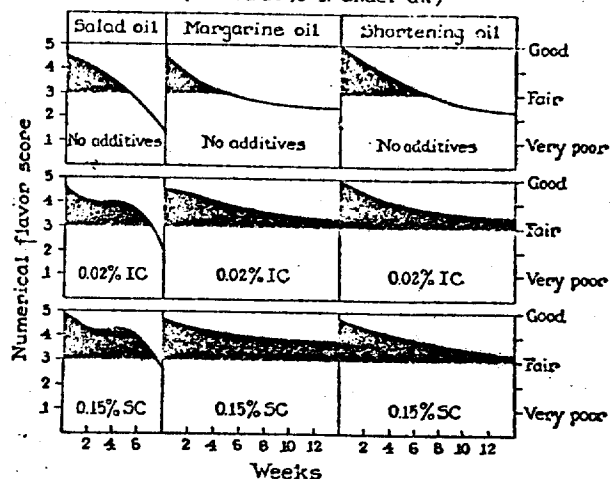
Figure 3. Value of commercially available esters of citric acid in stabilizing the flavor of limpid and hydrogenated soybean oil.

were plotted and the best-fitting curves drawn through the plot points. The point at which a curve crosses the horizontal line, representing a score of 3, i.e. an oil *fair* in flavor, represents the relative shelf life. In addition, the areas under the curves and above the "3" score line have been shaded to reflect not only the relative shelf life but also the degree of acceptability during the *better than fair* period. It is seen in Figure 3 that not only are the flavor lives of the oils extended by the use of the citrate esters, but also the "overall flavor" performances of the oils (as represented by the shaded areas) are markedly improved.

As shown in Table 1, the relative shelf life of the limpid soybean oil is extended by about 50% as a result of supplementation with the esters of citric acid and that of the hydrogenated oils more than doubled or tripled. The overall picture of flavor acceptance, as represented by the shaded areas in Figure 3, is shown numerically here to be improved by from 50 to 100% in the case of the limpid oil and by from at least 90 to over 300% in the case of the hydrogenated oils. In other words, the esters have also contributed to the maintenance of a higher degree of flavor acceptance during the period when the oils were satisfactory in flavor. Apparent shelf life based solely on peroxide value determinations in these same test systems seemed to indicate a 5-fold improvement. This comparison emphasizes that a more

TABLE 1

Performance of isopropyl and stearyl citrates in stabilizing the flavor of soybean oil stored under air at 95° F.

Oil	Additives	Relative shelf life [c] (weeks)	Overall flavor [d] performance
Salad oil	None	5	215
	0.02% IC	7	325
	0.15% SC	7½	425
Margarine oil	None	5	165
	0.02% IC	14+	550+
	0.15% SC	14+	700+
Shortening oil	None	7	315
	0.02% IC	14+	625+
	0.15% SC	14+	600+

[c] Number of weeks scored above *fair*.
[d] Relative shelf life × degree of acceptability; i.e., the area in sq. mm. above *fair* level under curve as originally plotted.

complete study of the flavor life of an edible oil is obtained by peroxide values supported by actual flavor scorings.

Tests using the A. O. M. method. The A. O. M. test is frequently used to evaluate antioxidants. This procedure has a very important use as a control method for routine production of a single class of oil products. However, A. O. M. stability tests are usually conducted in an all-glass apparatus on freshly processed oils. The citation of such data neglects the possibility

of changes in the values due to subsequent metal pickup. Oils are transported from refiner to bulk users in sheet iron tank cars. Users cream the fat with other ingredients in sheet iron mixers, or fry the products in sheet iron vats. Then, why conduct A. O. M. tests only in glass? A. O. M. tests on oils relatively free of metals can fail to predict which oil is better protected against subsequent metal pickup. In this laboratory, the A. O. M. Stability Test has been modified for investigative purposes by replacing the glass tubes with iron tubes of the same design. The advantages and limitations of the modified procedure as a research tool are fully discussed elsewhere (9). The usual peroxide value determinations are supplemented with flavor scorings of the oils as they undergo oxidation. For comparative purposes, tests using the all-glass apparatus are also conducted.

In Figure 4 are presented the results of a study of commercially available shortening products. With the exception of

Hours to rancid point (100 peroxide value)   Hours to rancid point (100 peroxide value)

Glass tubes   Iron tubes

A.O.M. test of shortenings

Code: A & B = Lards + antioxidant mixtures
C - K = Hydrogenated vegetable oils
L = Hydrogenated vegetable oil + 0.08% isopropyl citrate
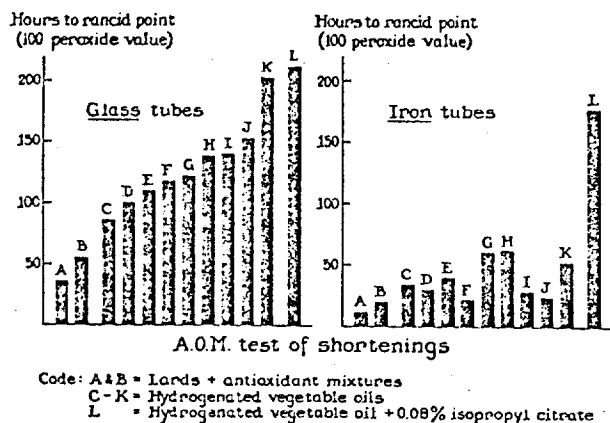
Figure 4. The marked decreases in A. O. M. stability values when shortenings are aerated in iron tubes.

Samples A and B which were lard products supplemented with antioxidants, all the other shortenings were hydrogenated vegetable oils. The striking decreases in the A. O. M. stability values when the tests are run in iron tubes are quite apparent in all samples except one. Only in the case of shortening "L," containing the relatively high level of isopropyl citrate, 0.08%, a concentration not obtainable with free citric acid, is the resistance to oxidation maintained to almost the same degree as that in glass tubes.

A comparable study, as exhibited in Figure 5, deals with the evaluation of antioxidants by means of the customary and the modified A. O. M. test. In this particular series, the same hydrogenated vegetable oil was used throughout, but supplemented with different antioxidants, alone and in combinations

Hours to rancid point (100 peroxide value)   Hours to rancid point (100 peroxide value)

Added antioxidants   Added antioxidants

Glass tubes   Iron tubes

A.O.M. tests of a vegetable shortening

Code: BHA = 0.02 % butylated hydroxyanisole
CA = 0.005 " citric acid
PG = 0.005 " propyl gallate
L = 0.10 " lecithin
IC = 0.05 " isopropyl citrate
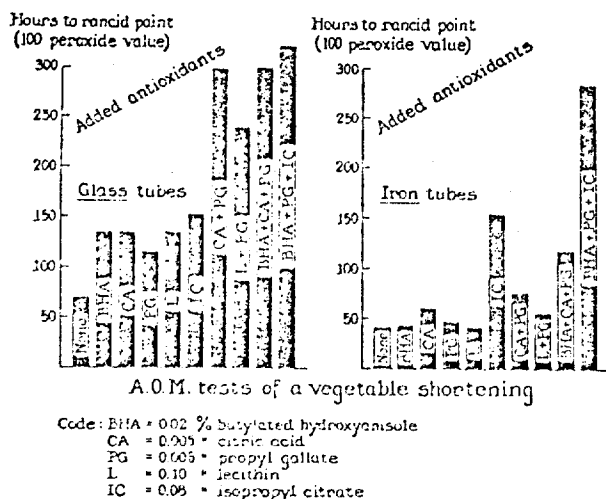
Figure 5. The effectiveness of isopropyl citrate, predominantly the mono-ester, in protecting shortening against oxidative deterioration accelerated by iron catalysis.

in the customary concentrations used commercially as indicated. From the results obtained in glass tubes, various antioxidant combinations are apparently superior to the isopropyl citrate used as sole protecting agent. However, when the tests are conducted in iron tubes, this is not the case. Under these accelerated conditions of test, providing for oxidative deterioration of the oils promoted by metal pickup and contact metal catalysis (9), conditions encountered during commercial operations, the relatively high concentration of isopropyl citrate confers a degree of stability strikingly superior to that attained with the other antioxidants, whether alone or in combinations. The best combination of antioxidants appears to be that including butylated hydroxy-anisole, propyl gallate, and isopropyl citrate. Flavor scorings conducted on all these test systems supported the A. O. M. values reported.

The results presented in Figures 4 and 5 are representative of a number of such studies. The precision of the tests conducted on oils and on antioxidants in iron tubes has been found to be almost as good as that obtained with the unmodified procedure (9). The values are reproducible in the authors' laboratory to within ± 2 hours. It is apparent from Figures 4 and 5 that citric acid itself cannot be readily incorporated in oils in the concentrations necessary to produce the effects obtainable with monoisopropyl citrate.

SUMMARY

The necessity of adding a metal-deactivating agent for stabilizing oils and fats against oxidation and flavor reversion is emphasized. Citric acid is an effective material, but in using this compound, the processor has no control over what remains qualitatively and quantitatively in the finished oil, not to mention the possibility of operational problems when the addition is made during the deodorization stage. These criticisms do not apply when the esters of citric acid are employed after deodorization. Ease of use and ready solubility very definitely favor the esters of citric acid. Far higher concentrations of metal-deactivator compounds in oils are readily attained with the esters of citric acid for counteracting the pro-oxidant effects of metals. This is illustrated by results obtained from a modified A. O. M. test procedure involving aeration of oils in iron tubes rather than in glass.

The plea is made that objective flavor scorings be carried out more often on oil and fat products held under realistic conditions of storage before generalizations on flavor life are drawn. Peroxide value determinations are of greater value when supplemented by such flavor scorings.

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the L(−) salt by fractional crystn. The free acids are obtained by acidification of the resp. salts. Thus, a mixt. of 1 mole (−)-ephedrine and 1 mole I was dissolved in 337 ml. hot amyl acetate, filtered to remove insol. impurities, and finally chilled for 2 hrs. The yield of cryst. solids collected after filtration and washing with fresh solvent and drying was 169 g. (93%). $[\alpha]_D^{25}$ −9.1°. The salt (165 g.) was recrystd. from 297 ml. hot amyl acetate and isolated to give 124 g., $[\alpha]_D^{25}$ −4.2°. Further recrystn. using the same solvent (1.8 ml./g.) gave 110 g. (88%) of a salt with $[\alpha]_D^{25}$ −3.2°. The salt (100 g.) was suspended in 500 ml. H₂O and 500 ml. ether and acidified. The ether was sepd. and the aq. phase extd. with 250 ml. ether. The combined ether exts. were washed with H₂O and extd. with NaHCO₃ soln. The alk. ext. was then acidified and chilled. The amt. of pptd. D(+) acid collected was 47 g. (90%) with $[\alpha]_D^{25}$ +50.5°. Similarly, L(−)-α-phenoxybutyric acid was isolated in 90% yields starting from 1 mole I and 1 mole cinchonidine; $[\alpha]_D^{25}$ −50.1°.

Juan Longoria III

Prophylaxis against smallpox and treatment of vaccinia with 1-methylisatin β-thiosemicarbazone. Denis J. Bauer and Peter W. Sadler (to Burroughs Wellcome & Co. (U.S.A.) Inc.). U.S. 3,253,991 (Cl. 167–65), May 31, 1966; Appl. Dec. 5, 1960, and Nov. 22, 1963; 4 pp. 1-Methylisatin β-thiosemicarbazone (I), m. 245°, is effective in the prophylaxis and treatment of smallpox and vaccinia. Also prepd. were the p-thiosemicarbazones of the following substituted isatins (substituent and m.ps. given of isatin and thiosemicarbazone): 1-Et. —, 204°; 1-Pr, —, 193°; 1-Bu, 36°, 155°; 1-pentyl, 47°, 184°; 1-iso-Pr, ~62°, 225°; 1-(prop-2-ynyl), 163°, 250° (decompn.); 1-allyl, 89°, 201°; 1-hydroxymethyl, —, 230°; 1-(β-hydroxyethyl), 118°, 247°; 1-acetoxymethyl, 109°, 230°; 1-(2-acetoxymethyl), 113°, 244° (decompn.); 1-(2-bromoethyl), 131°, 227° (decompn.); 1-acetyl, —, 244°; 5-fluoro-1-methyl, 151°, 260°; 1-ethyl-5-fluoro, 131°, 228°; 1,7-trimethylene, 191°, 236°; 1,7-diethyl, —, 258° (decompn.).

Joachim Anschel

Germicides. Whitmoyer-Reed Ltd. Belg. 672,353, March 16, 1966; Brit. Appl. Nov. 16, 1964; 12 pp. Title compns., as a concentrate for diln. with H₂O, contain iodine, an amphoteric H₂O-sol. surfactant R¹R²R³CNH(CH₂CHR⁴O)ₙSO₃X and H₃PO₄. The concentrate contains 0.5 to 6% of iodine and with the proportion of surfactant from 2:1 to 10:1 and 2 to 5% 85% H₃PO₄. For example, 3.5 parts of iodine and 20 parts of the surfactant (X = Na, R⁴ = H, n = 15 and the tertiary amine radical contained 12 to 15 C atoms) were intimately mixed to form a homogeneous iodophore which can be transformed into a germicidal concentrate suitable for the dairy industry and for the breeding of poultry. Then, 23.5 parts of the iodophore was mixed with 50 parts of 85% H₃PO₄ and with H₂O to give 125 parts total. The diluted product formed an excellent detergent germicide.

F. J. Sprules

Germicides containing iodine and a surfactant. Michel Plissier. Fr. 1,426,139 (Cl. A 61l), Jan. 28, 1966, Appl. July 7, 1964; 8 pp. Title compns. are prepd. by warming an iodine complex and an anionic surfactant such as an alkali metal or ammonium salt of a sulfated fatty alc. contg. 8 to 18 C atoms, preferably sodium lauryl sulfate. The two materials are warmed in a sealed vessel without H₂O. Later, H₂O is added, the pH adjusted to 8, and this mixt. warmed further. In the first stage, the warming is done at 60–80° for 1–3 hrs. In the second stage, warming lasts 12–24 hrs. The ratio of iodine to surfactant is 1:20 to 1:40. For each g. of iodine used, 100 cc. H₂O is added. The new compns. thus contain 0.5 to 0.7% iodine. The ratio of surfactant to iodine varies from 20:0.5 to 40:0.7. From 0.25 to 0.65% of the iodine is combined. F. J. Sprules

Antithrombose preparations. Aktieselskabet Trombo. Neth. Appl. 6,507,473 (Cl. A 61k), Dec. 13, 1965; Brit. Appl. June 11, 1964; 18 pp. The active component of the prepn. is linolenic acid (I). It is used as its Ca or Mg salt, or it can be added to edible oils and fats such as butter; in this case, antioxidants have to be added. The Ca salt of mixed fatty acids contg. I are prepd. as follows: 10 kg. refined I is refluxed 30 min. with 70 ml. 0.5N alc. KOH. The mixt. is cooled to ambient temp., and 140 ml. 0.25N Ca(OAc)₂ soln. in 70% EtOH is added. The ppt. is sepd. and washed with 96% EtOH to give 7.2 g. of the mixed Ca soap (fraction 1). The EtOH is evapd. in vacuo from the filtrate and the residue washed with 300 ml. redistd. H₂O, filtered, and washed with H₂O to give 3.2 g. mixed Ca soap (fraction 2). Gas chromatographic anal. shows fractions 1 and 2 to contain 41 and 60.8% I, resp. A soln. of 2 g. I in 5 ml. H₂O is added to a soln. of 0.78 g. KOH in 20 ml. EtOH. At ambient temp., a 2M aq. soln. of CaCl₂ is added, and the ppt. is sepd. and worked up to give 2.16 g. I Ca salt.1/2Ca(OH)₂ (m. 160–3° (decompn.)). In an analogous manner, the I Mg salt.1/2Mg-(OH)₂ (m. 184–6° (decompn.)) is obtained. A prepn. contg. 800 g. margarine, 50 g. purified I, 0.06% tocopherol, 0.02% butylated hydroxyanisole (II), 0.02% ascorbyl palmitate (III), and 0.02% iso-Pr citrate (calcd. on I), and tablets of I Ca salt 650, DL-α-tocopherol acetate 0.3, Pr gallate 0.1, II 0.06, III 0.15, dried starch 200, mannitol 100, Et cellulose 1, and dried potato starch 49 mg., are described. S. A. V. Walle

Solid compositions containing salts of sulfated sulfonium inner salts. Imperial Chemical Industries Ltd. (by Alistair I. Reid, Hugh M. Young, and Walter C. McArthur). Brit. 1,033,418 (Cl. C 07c), June 22, 1966, Appl. Nov. 2, 1962; 2 pp. An aq. soln. contg. 18–20% disodium tris(β-sulfatoethyl)sulfonium inner salt ⁻O₃SOCH₂CH₂S⁺(CH₂CH₂OSO₃Na)₂ (I) and 8% Na₂SO₄ was fed at ambient temp. to a lab. spray drying app. equipped with a nozzle, at a feed rate of 200 ml./hr. The inlet air temp. was 80–1000°, and chamber temp. 65–70°. Air passing through the app. at 550–600 ft.³/min. gave a compn. contg. 58–82% I, which is useful as a bactericide. Sister Miriam Grace Solomon

Stable adenosine triphosphate derivative. Morishita Pharmaceutical Co., Ltd. (by Shigesaburo Takenaka). Japan. 7473('66) (Cl. 30 C 2), April 22, Appl. Dec. 10, 1963; 2 pp. Adenosine triphosphate (ATP) (1 mole) is stirred at 40° for 15 min. with 4 moles cysteine and 1 mole α-oxoglutaric acid, the soln. neutralized, stirred with CaCl₂, 21 moles MeOH (or Me₂CO) added, and the mixt. centrifuged to give the Ca salt (I) of ATP-cysteine, hardly sol. in H₂O, non-hygroscopic, and useful as a coronary vasodilator. Hiroshi Kataoka

Pharmaceuticals containing dimethyl sulfoxide. Olin Mathieson Chemical Corp. Belg. 670,560, April 6, 1966, Appl. Oct. 6, 1965; 9 pp. Pharmaceuticals were prepd. contg. 10.0–99.8% Me₂SO, 0.25–2.0% gelling agent, and 0.01–20% therapeutic drug. Me₂SO was mixed with a carboxyvinyl polymer vehicle and stirred with N(CH₂CH₂OH)₃ to give a gel contg. Me₂SO. Other gels were prepd. using triamcinolone acetonide and hydroxychloroquine. Judith A. Douville

Urea-containing diuretics. CIMEPHA S.A. Brit. 1,014,233 (Cl. A 61k), Dec. 22, 1965; Fr. Appl. Jan. 23, 1963; 13 pp. An aq. soln. of 30% urea and 10% mannitol injected intravenously has a diuretic and cerebral decompression effect. Joachim Anschel

Pharmaceutical composition for treating rheumatoid arthritis. Eustace C. Barton-Wright. Brit. 1,033,843 (Cl. A 61k), June 22, 1966, Appl. May 20, 1964; 2 pp. The oral administration of a compn. contg. d-pantothenic acid and 10-hydroxy-Δ¹-decenoic acid gave considerable remission of the symptoms of rheumatoid arthritis as evidenced by tests on human beings. Acceptable salts of these acids could be used admixed with an excipient, such as lactose or starch, and this compn. placed in an enteric container. Thus, 50 mg. Ca d-pantothenate, 5 mg. 10-hydroxy-Δ¹-decenoic acid, and 45 mg. lactose were mixed and filled into hard gelatin capsules, and the capsules were enteric-coated with cellulose acetate phthalate or keratin.

George Meister

Surgical skin disinfectant. Koninklijke Industriele Maatschappij voorheen Noury & van der Lande N.V. Neth. Appl. 6,410,461 (Cl. A 61l), March 11, 1966, Appl. Sept. 9, 1964; 3 pp. A soln. of an iodine-poly(N-vinylpyrrolidinone) adduct (I) in 96% EtOH contg. ~1% tartaric acid (II) was used as a preoperative surgical skin disinfectant. Thus, I 1250 and II 450 g. were added to 45 l. 96% EtOH while stirring, and the vol. was brought to 50 l. with 96% EtOH. E. A. Gryp

Recovery of methionine from an aqueous solution containing ammonium sulfate and methionine. Stamicarbon N.V. Neth. Appl. 6,411,467 (Cl. C 07c), April 4, 1966, Appl. Oct. 2, 1964; 4 pp. The mother liquor (1 kg.) (contg. 35 wt. % (NH₄)₂SO₄ (I) and 1.4 wt. % methionine (II)), obtained after sepn. of II from a reaction product by crystn., was extd. with 617 g. 95 wt. % alc. at 70° to give 808 g. H₂O layer and 809 g. alc. layer. Upon removal of the alc. (29.5 g. and 587.5 g., resp., 95 wt. %), which was recycled to the extractor, 763.5 g. aq. soln. contg. 342 g. I and 236.5 g. aq. soln. contg. 14 g. II and 8 g. I were obtained. The latter soln. was cooled to 0° and filtered to give 10.5 g. II, while the filtrate was returned to the reactor.

Jack J. Weber

Oral contraceptive composition. SPOFA United Pharmaceutical Works. Neth. Appl. 6,512,823 (Cl. A 61k), April 7, 1966; Czech. Appl. Oct. 6, 1964; 3 pp. A contraceptive is prepd. contg. an estrogen and a gestagen component in amts. which are equiv. to 2000 I.U. estradiol and 10 I.U. progesterone with an admissible difference of 5%. Such prepns., e.g. contg. 0.1 mg. ethynylestradiol 3-Me ether and 5 mg. 16-methylene-6-dehydro-17α-acetoxyprogesterone in a daily dose, do not have an androgen side-action and do not damage the blood vessels or the liver function.

P. Vink

Antivomiting agents for children. Berk Pharmaceuticals Ltd. Brit. 1,032,175 (Cl. A 61k), June 8, 1966; Fr. Appl. July 19, 1962; 4 pp. A medicament for inhibiting vomiting in babies and young children comprises 5–30% of a trimethylsilyl-terminated poly(dimethylsiloxane) (I) of d. 0.973 and n_D 1.4035, and contg. ~330 SiMe₂O units, in whole carob bean flour (II). A typical compn. contains 10 g. I, 50 g. II, and sweetened excipient to 100 g. The mixt. is granulated, dried and finely sieved. The compn. is useful in preventing vomiting due to infections such as meningitis, surgery, pyloric stenosis, lactation intolerances, etc.

P. Mamalis

Complex of chlorhydroxyquinoline with bismuth. Olin Ma-

Effect of Antioxidants and Synergists on Peroxide Decomposition in Milk Fat [1]

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Abstract

Antioxidants markedly accelerate milk fat peroxide breakdown in vacuo at 40 C. Except for nordihydroguaiaretic acid the rate of breakdown of peroxides by the antioxidants increases with increasing antioxidant concentration. With nordihydroguaiaretic acid, the rate of breakdown decreased with increasing nordihydroguaiaretic acid concentration. The synergists, citric acid and isopropyl citrate, decreased the rate of breakdown of peroxides compared with controls and significantly inhibited the acceleration of peroxide decomposition caused by the addition of antioxidants.

Introduction

Antioxidants are widely used to increase the stability of fats and oils. Their effectiveness is generally attributed to their ability to react with free radicals and terminate the chain reaction between unsaturated fatty acids and oxygen (7). Certain substances called synergists are known to enhance the effectiveness of antioxidants. Some investigators have postulated that synergists react with traces of metal ions in the system and prevent the metal ions from catalyzing the oxidation (7). There is no doubt that metal ions will catalyze autoxidation and that it is extremely difficult to remove the last traces of them from fatty materials.

Privett (3) and Privett and Quackenbush (4,5) studied the effect of antioxidants on the decomposition of peroxides in the absence of oxygen, and they found that α-tocopherol, NDGA, and hydroquinone greatly accelerated the decomposition of peroxides in lard incubated at 100 C. The synergists, ascorbic acid and citric acid, when added along with the antioxidants, inhibited their ability to decompose peroxides. This suggests that synergists

have an effect on antioxidants that may not be mediated by metal ions.

The purpose of this paper is to confirm the interesting observations of Privett and Quackenbush, to extend their observations to milk fat and the low ranges of peroxide values important in organoleptic studies, and to see if the effect they reported is useful in the selection of antioxidant-synergist combinations for the preservation of milk fat.

Experimental Methods

Milk fat was prepared as described previously (2) and allowed to oxidize to a peroxide value of one to two. The oxidized oil was then subjected to a vacuum of 5μ or less for at least 12 hr. Then, with as little exposure to air as possible, the fat was divided into four parts, and ethanol solutions of antioxidants were added to three of the portions of fat. An equal volume of ethanol was added to the fourth portion as a control. The four portions of fat were degassed for 30 min under vacuum to remove most of the alcohol, and the samples were transferred by syringes to a number of small tubes. The tubes were constricted to make it easier to seal them under vacuum. Exposure to air again was avoided as much as possible during the transfer. The tubes were degassed for 24 hr under a vacuum of $5\ \mu$ or less to remove the last traces of ethanol and oxygen. The tubes were sealed with a hand torch while still under vacuum.

The sealed tubes of oxidized fat were incubated in a water bath at 40 C. At suitable intervals tubes were removed to determine the peroxide value by the method of Hamm et al. (1).

Results and Discussion

The magnitude of the antioxidant effect varied with the particular lot of milk fat, and for this reason the antioxidants BHA, NDGA, and α-tocopherol were compared using the same lot of milk fat. The tests were made with addition of both 0.1 and 1.0% antioxidant. Results are given in Figures 1 and 2. Nordihydroguaiaretic acid had the least effect at 1%, but at 0.1% it had a greater effect than BHA
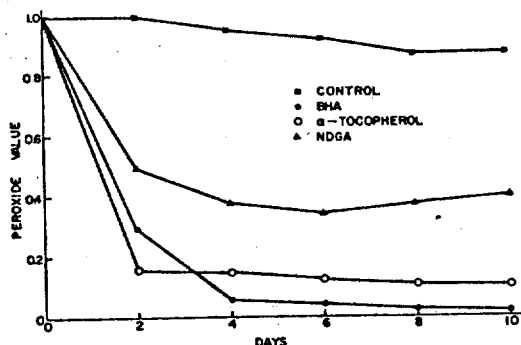
FIG. 1. Comparison of the effectiveness of anti-oxidants at the 1% level on the decomposition of milk fat peroxide in vacuo.

and about the same effect as α-tocopherol. Addition of 0.05 and 0.01% antioxidant was tried, but the effect on peroxide decomposition was too small to be observed consistently. Thus, these effects can only be observed well above the legal limit of 0.02% by weight of antioxidant. The level of α-tocopherol expected in milk fat is only about 0.0024%; therefore the addition of antioxidant at 0.01 or 0.05% should be a considerable increase over the natural level. In their studies on lard, Privett and Quackenbush (3–5) used much higher peroxide values and suggested that the antioxidant might be catalytic. In our experiments the molar ratio of antioxidant to peroxide was about 40 to 1 at the 1% level of antioxidant and 4 to 1 at the 0.1% level, so it would be impossible to distinguish a catalytic effect from a stoichiometric one.
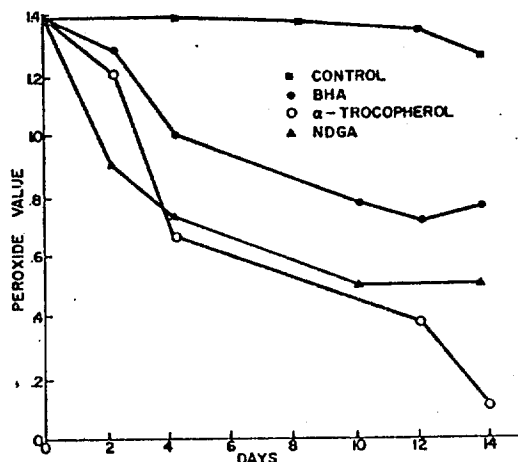
The effect of 1% of the synergist isopropyl

FIG. 2. Comparison of the effectiveness of anti-oxidants at the 0.1% level on the decomposition of milk fat peroxide in vacuo.
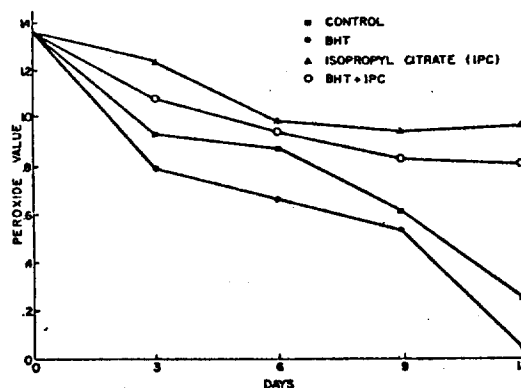

FIG. 3. Effect of 1% butylated hydroxytoluene (BHT), 1% isopropyl citrate (IPC), and 1% BHT + 1% IPC on the decomposition of milk fat peroxides in vacuo.

citrate is shown in Figures 3 and 4. In some instances, the rate of peroxide breakdown in samples treated with synergist was less than that of the control. This same effect was found with citric acid as a synergist. The combination of synergist and antioxidant gave considerably lower rates of peroxide decomposition than samples containing only antioxidants, and in some instances lower than the control.

These results completely confirm the findings of Privett and Quackenbush and show their conclusions are also valid for other antioxidants and synergists.

Since the synergist alone seems to stabilize the peroxide, it is likely that the synergist is binding traces of metal ions present in the fat and preventing these ions from catalyzing peroxide decomposition. The synergist may block peroxide decomposition by inhibiting peroxide decomposition by the small amount of tocopherol naturally present in the milk fat; however, our results indicate that the effect
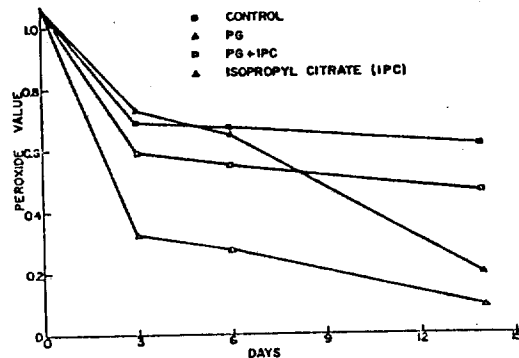

FIG. 4. Effect of 1% propyl gallate (PG), 1% isopropyl citrate (IPC), and 1% PG + 1% IPC on milk fat peroxide decomposition in vacuo.

of this amount of tocopherol on peroxide re-composition would be too small to observe.

The synergists also blocked the acceleration of peroxide decomposition caused by the addition of antioxidants. One could argue that metal ions are required for the accelerating action of the antioxidants, and the effect of the synergist is still a metal ion effect. However, antioxidants, synergists, and peroxides are all considerably more polar than the fatty medium in which they are dissolved. Under such conditions they might be expected to associate. The association of peroxides has already been invoked to explain certain kinetic phenomena (6). The synergist may simply be associating with the antioxidant or peroxide and decreasing their association with each other.

If the oxidized flavor of milk fat arises from the decomposition of peroxides, as is commonly assumed, then the addition of synergist to antioxidants ought to prevent oxidized flavor much better than antioxidants alone. Recent work at our laboratory indicates that this is not true for isopropyl citrate (2). This may be because the oxidized flavor arises primarily by the breakdown of free radicals during the chain reaction before hydroperoxides are formed. The effects noticed at 0.1% antioxidant level may not occur at the 0.02% level used in the organoleptic experiments. The effect of antioxidants and synergists may not be the same on all fatty acid peroxides; therefore, a general measure of peroxide value does not tell the complete story. Although isopropyl citrate was not particularly effective in suppressing oxidized flavors, thiodipropionic acid which is usually regarded as a synergist was quite effective.

The samples for peroxide values at zero time were taken just after the sealing of the tube, after they had been degassed in the presence of antioxidants for about 12 hr. Nearly always the control sample had a higher peroxide value than the samples treated with antioxidant. The peroxide value by the method of Hamm et al. (1) is an empirical procedure. To make sure that the peroxide values recorded were not being affected by the large amounts of antioxidant being added, samples of milk fat were treated with amounts of antioxidant ranging from 0.01 to 3.2%, and the peroxide values were taken immediately after addition of the antioxidant. The peroxide values all agreed with the control, indicating that the antioxidant had no effect on the test, and the decrease in peroxide during degassing in the samples treated with antioxidant was due to the acceleration of peroxide breakdown by the antioxidants.

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